HEALTH AND PERFORMANCE OF RANCHED SOUTHERN BLUEFIN TUNA

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STATEMENT OF CO-AUTHORSHIP

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ABSTRACT

The Australian southern bluefin tuna (SBT) industry is continually looking for ways to improve fish health within the ranching environment, both for increased profitability and concern for animal welfare. This thesis utilized current and newly acquired knowledge about southern bluefin tuna health to make educated manipulations of their ranching environment and/or husbandry practices. SBT health, parasites and performance where then monitored as a measurement of success. Five major projects were completed, all projects completed on the commercial scale. Three projects focused on environmental and/or husbandry manipulations: (chapter 2) dietary supplementation of immunostimulants and vitamins; (chapter 4) moving cages further offshore; (chapter 6) an assessment of two management strategies for blood fluke, *Cardicola forsteri*, infection. While two projects aimed to gain more information about ranched SBT health: (chapter 3) a detailed description of the first two months of ranching; (chapter 5) correlation of SBT humoral immune response with infection stage of *C. forsteri*.

Greater knowledge was obtained relating to the effects of ranching. Over the first two months, weight, length, condition index, hemaoglobin concentration, and immune response were all found to change significantly. Ranched SBT were found to acclimate to ranching within one month post transfer and were relatively healthy prior to an acute mortality event from week five to seven of ranching, which resulted in a cumulative mortality of 8.5%. The mortality event was associated with decreased hemaoglobin concentrations and changes in immune response. Additional information was also gained on one of the most common and significant infections during ranching, blood fluke *Cardicola forsteri.* The timeline for *C. forsteri* infection was validated using natural infections in SBT. Humoral immune response (i.e. lysozyme, alternative complement and specific antibody activity) was correlated to infection and was found to develop concurrently with *C. forsteri*. The majority of physiological response coincided with commencing egg production, at approximately 5 to 7 weeks of ranching. New and previous knowledge regarding *C. forsteri* infection in SBT were merged, resulting in a inclusive infection, physiological response and diagnosis timeline.

Further enhancement of ranched SBT health and performance was also obtained within this thesis utilizing dietary supplementation, adjustments in ranching location, and chemotherapeutics. Supplementing the diet of ranched SBT for the first twelve weeks with Vitamins E and C resulted in 1.5 times higher lysozyme activity at 8 weeks of ranching. Vitamin supplementation was also associated with tow specific improvements in performance, including enhanced survival, decreased *Cardicola forsteri* prevalence and intensity, and enhanced alternative complement activity. No changes in health, immune response, and performance were associated with immunostimulant supplementation. Examination of the feasibility of offshore ranching yielded significant effects on the health and performance of ranched SBT. Compared to SBT ranched in the traditional near shore environment, SBT ranched further offshore had enhanced survival, increased condition index at 6 weeks of ranching, and superior health. The offshore cohort had no *C.forsteri* and a 5% prevalence of *Caligus* spp. compared to a prevalence of 85% for *C. forsteri* and 55% prevalence for *Caligus* spp. near shore at 6 weeks of ranching. In addition, offshore ranched SBT had elevated hemaoglobin concentration and lysozyme activity. Finally, management of *C. forsteri* infection was examined utilizing two management strategies: (1) chemotherapeutic treatment with Praziquantel and (2) temporary offshore ranching. Both management strategies successfully reduced infection as well as mortality, yet evidence of reinfection and/or delayed infection suggest further research needs to be completed to optimize these strategies.

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NOTE TO READERS:

THIS THESIS IS BASED ON A COLLECTION OF PUBLISHED PAPERS; THEREFORE SOME REPETITION BETWEEN CHAPTERS OCCURS.

"Besides high prices, the bluefin commands an awed, almost mystical respect and devotion among those who know the animal intimately. One says, "If you talk to enough fishermen, you may sense how much we really love the bluefin and how much they mean to us. I think it's the way the Indians felt about the buffalo." But people love the bluefin in different ways. Fishers, conservationists, governments, and international treaty organizations continually embroil themselves in bitter international struggles over control and salvation of the fishery."

Carl Safina, *Song for the Blue Ocean*

CHAPTER 1:

GENERAL INTRODUCTION

Bluefin tuna are one of the most valuable and unique fish in the world. Biologically, they have the highest metabolic rate and digestion rate of any fish, they have a respiratory capacity approaching that of mammals, and they are the only truly warm blooded fish. Economically, bluefin tuna are so valuable they are sometimes nicknamed 'living gold', with the commercial and recreational fisheries producing thousands of jobs worldwide. There are three species of bluefin tuna, one for each major world ocean: Atlantic bluefin tuna (ABT) *Thunnus thynnus* (Linnaeus 1758), Pacific bluefin tuna (PBT) *Thunnus orientalis*, (Temminck and Schlegel 1844) and Southern bluefin tuna (SBT) *Thunnus maccoyii* (Castelnau 1872). This thesis will focus on Australian SBT exclusively.

1.1 SBT Commercial Background

1.1.1 Fishery

Large scale commercial fishing for SBT began in the early 1950s by Japan and Australia (Mori *et al.* 2001, Polacheck *et al.* 2004), utilizing both surface poling and deep-sea longlining. Fishing covered a wide area, with the Japanese fleet expanding from the spawning grounds near Java (Polacheck *et al.* 2004) to just west of New Zealand in 1959 (Hearn *et al.* 2003), and the Australian fleet targeting the southeast coast of Western Australia and the southern coast of New South Wales (Hearn *et al.* 2003). The largest commercial catch recorded was 81,605 tons in 1961. Since then, commercial catch and catch rate have declined (Polacheck *et al.* 2004), yet commercial effort exponentially increased. In the 1970s, four more nations entered the fishery: New Zealand, Taiwan, Indonesia, and Korea. The first evidence of overfishing was observed in the 1980s, when the surface fishery off the southeast coast of Australia suddenly collapsed (Polacheck *et al.* 2004). Tagging experiments correlated the collapse to higher juvenile exploitation (Caton 1991). In response, Australia, New Zealand and Japan decided to reduce their fishing effort voluntarily, resulting in a drastic reduction to ~50% catch (Polacheck *et al.* 2004). Although catch was significantly reduced, stock assessments through the mid-1990s showed a continuous and substantial decline in the population *(*Anonymous 1994*,* Kolody *et al.* 2001*,* Polacheck *et al.* 2001). By 1990, the spawning stock of SBT was estimated at 6-11% of the 1960 size (Grewe *et al.* 1997, Kolody *et al.* 2001, Polacheck *et al.* 2001, Ward 1995), so small it was considered an endangered

species (Safina 1995). In response to the dire status of the fishery, catch quota was further reduced by 70% of its previous yearly tonnage (CCSBT 2008) and in 1994, the Commission for the Conservation of Southern Bluefin Tuna (CCSBT) was established by Australia, New Zealand, and Japan. The goal of this organization is to recover spawning stock biomass to the 1980s level by 2020 (CCSBT 2008). Catch is currently managed by individual transferable quotas (ITQ), with the total allowable catch (TAC) reassessed each year (Clear *et al.* 1999). Some stock recovery has been recorded since conservation begin, yet catch remains substantially restricted and only a fraction the size of the pre-1990s level (Polacheck *et al.* 1997).

1.1.2 Ranching

In response to dwindling stocks, limited quota and an uncertain economic future, ranching began in Port Lincoln, SA in 1991. The goal of ranching was to optimize production efficiencies, attend to fish welfare, and enhance market values in order to maintain sustainability and profitability, thereby saving the fishery, its jobs, and its cultural importance to the Port Lincoln community. Ranching also allowed the Australian industry to break into the highly valued sashimi market, previously unattainable due to the long delay in travel time between wild catch and market and a low to average flesh quality. Today, the SBT ranching industry is one of the most successful in Australia, valued at over AU$300 million, and is an important employer in the Eyre Peninsula region of South Australia (Gardner *et al.* 2006).

Ranching is based on purse seining large schools of 2-4 year old juveniles as they migrate eastward through the Great Australian Bight each year. SBT are transferred from purse seine to a towing cage and then towed into the Tuna Offshore Farming Zone of Port Lincoln, S.A. When fish arrive within the zone, they are transferred into several grow-out cages (also known as pontoons) and ranching officially begins. In ranching, fish are held for three to six months while being fattened on baitfish, not only putting kilos on the fish, thereby increasing meat yield, but also increasing their value in the market place through increasing fat content. Value with tuna is assessed by a number of parameters, the top among them being size and fat content. Ranching allows fish to be harvested on demand, for either the fresh or frozen market, thereby giving the industry an edge on the market.

Since its development, bluefin tuna ranching quickly spread across the world, with operations now also existing in the Mediterranean, Mexico, and Japan. Continued growth is expected as husbandry and technological advances improve survival, fattening efficiency, allow for extended holding times, and creation of more proficient farming technologies.

1.2 SBT Biology

1.2.1 Life History

Southern Bluefin Tuna (SBT) exist as one continuous population; occurring from the tropical to temperate waters of the Indian Ocean between Java and north-western Australia (Grewe *et al.* 1997, Ward 1995). Mean age at maturity ranges between 8 (Jessica *et al.* 1998) to 12 years (Davis *et al.* 1998). Spawning occurs off the Northwest coast of Australia, south of Java, between September and April, and is not synchronous for the whole population, with peaks occurring in October and February. This may be due to either the widespread distribution of adults in the feeding grounds (Farley *et al.* 1998) or because of age, with younger individuals spawning earlier in the season than older individuals (Grewe *et al.* 1997). Throughout the spawning season, new spawners continuously replace old spawners on the spawning ground (Farley *et al.* 1998). SBT females can spawn several times in one season, the average interval between batches is 1.1 days, with fecundity increasing with body length. Adults rapidly leave the spawning grounds post spawning due to either decreased food availability or their inability to withstand the warmer tropical temperatures for long periods of time (Farley *et al.* 1998).

High tropical temperatures, averaging 27°C (Sharp *et al.* 1978), are required for embryo and larval development up to the first year when their counter current heat exchangers are fully developed (Sharp *et al.* 1978). Within a few months of hatching, juveniles migrate south along the continental shelf, assisted by the Leeuwin Current (Maxwell *et al.* 1981). After one year post hatch, juveniles first appear in the Great Australian Bight, and slowly begin to disperse south toward New Zealand or west to south Africa from two to four years of age (Grewe *et al.* 1997). Soon after spawning, adults also migrate from either the western coast of Australia to New Zealand and partially up the east coast of Australia or towards South Africa where they gain condition in the rich southern waters (Mori *et al.* 2001).

SBT undergo two different growth/behavioral periods; the first from hatch until 80 cm when they are found in tight schools located in nearshore waters and the second from 80cm to adult when they move independently offshore (Hearn *et al.* 2003). Intra-annual variation also occurs, with the fastest growth occurring during the Austral summer, between January and March (Polacheck *et al.* 2004) and the slowest growth occurring during the Austral winter between July and September (Rooker *et al.* 2007). Hypothesis for the change in growth rate included changes in temperature, food, spawning condition, and stress (Bayliff 1993, Mather *et al.* 1960). By age of 10 year, females are larger than males (Rivas 1976). SBT can live to 40 years (Hayes 1997) or more (Kalish *et al.* 1996).

1.2.2 Physiology

Bluefin tuna have the highest metabolic rates of any fish species (Brill *et al.* 1991). They also have the highest digestion rates and hemoglobin levels (Bushnell *et al.* 1994), with the respiratory capabilities of mammals (Dizon *et al.* 1978). They are unique among teleosts as having the most evolved endothermy, also approaching that of mammals (Stevens *et al.* 1978), within the muscle, viscera, and brain. It is believed that the increase in temperature is a byproduct of their increased metabolism (Brill 1996) and is maintained by counter current heat exchangers called rete mirabile (Stevens *et al.* 1978). The benefits of homeothermy include an expanded thermal niche (Brill 1996) and enhanced immune response at low temperatures (Watts *et al.* 2002).

1.2.3 Immune system

The immune system of fish can be divided into the innate (non-specific) and the acquired (specific) immune system. The components of the innate immune system of fish include cellular (i.e. monocytes, non-specific cytotoxic cells, neutrophils, eosinophils and macrophages) and humoral factors (i.e. transferrin, interferon, protease inhibitors, lysozyme, chitinase, agglutinins, precipitins, pentraxins, natural antibodies, cytokines, chemokines, and antibacterial peptides) (Magnadóttir 2006). The acquired immune system includes cellular (i.e. lymphocyte) and humoral (i.e. specific antibodies) factors (Kaattari & Piganelli 1996). Increasing evidence suggests the two systems are intimately intertwined, with the innate response activating and determining the nature of the acquired response (Fearon & Locksley 1996,

Fearon 1997). The major immune organs of teleost fish are kidney, thymus, and spleen (van Muiswinkel *et al.* 1991; Fänge 1994; Manning 1994).

The immune system of SBT is well developed compared to other fish, perhaps due to their endothermy. Larval development of the immune organs, at least in PBT, occurs more rapidly than all other marine fish, with innate immune response possible as early as 36 hours post hatch and humoral immune response as early as 7 days post hatch (Watts *et al.* 2003). In addition, unlike other fish species, SBT do not experience seasonal immunosuppression (i.e. total immunoglobulin, complement activity and lysozyme concentration) at lower water temperatures (Watts *et al.* 2002).

1.3 SBT threats to health

Very few disease problems have been reported in juvenile (2-4 years) and adult SBT. They are relatively resistant to bacterial infections even after trauma (Munday *et al.* 2003) and to date no viral diseases have been detected in wild or ranched SBT. Yet, over the years low levels of unexplained mortality have been observed within the commercial SBT ranching cages. Changes in farming techniques which reduce stress at capture, transport and holding have reduced overall mortalities, however there is still room for improvement as SBT ranching intensifies. Currently the highest ranked risks to SBT health are parasitic infections and chronic stress (Nowak *et al.* 2006).

1.3.1 Stress

Mortality can be needlessly high in farmed fish that are held in cages such as SBT. They are subjected to various kinds of stress, which could lead to immunosuppression, and are constantly exposed to potentially pathogenic microbes which are present in the water. Most disease outbreaks occur when immunosupression and pathogen exposure are present at the same time. Reduction of stress is also important out of concern for animal welfare. There have been several common stress responses used as indicators of stress in fish (Table 1.1), the most common among them being plasma glucose, hemaoglobin, plasma osmolality, and physical condition indices such as condition factor, growth, and organosomatic indices (Morgan & Iwama 1997). Stress has been managed in several ways in ranching, including maintaining slow speeds during tow, reducing stocking density, using frozen block feeding

cages to maintain feed throughout the day, and locating cages in areas with high water currents and good water quality. Although stress management has been significantly addressed, there is always room for improvement as new sources of stress are discovered.

Table 1.1 Stress responses commonly used as indicators of stress in fish. [Modified from Barton *et al.* (1997) and Shreck *et al.* (1997).]

Primary Stress Response (Endocrine level)	Plasma catecholamines	
	Plasma cortisol	
Secondary Stress Response (Metabolism and Osmoregulation)	Metabolic	Plasma Glucose
		Plasma Lactic acid
		Plasma cholesterol
		Liver and muscle glycogen
		Liver and muscle adenylate energy charge
	Haematological	Haematocrit
		Leucocrit
		Erythrocyte (RBC) count
		Leucocyte count
		Lymphocyte: RBC ratio
		Thrombocyte count
		Blood clotting time
		Hemaoglobin
	Hydromineral	Plasma Cl
		Plasma Na
		Plasma K
		Plasma protein
		Plasma osmolality
	Structural	Interrenal size and number
		Interrenal cell nuclear diameter
		Gastric tissue morphology
		Organosomatic indices
		Condition factor
Tertiary Stress Response (Behavior)	Activity	
	Swimming performance	
	Schooling	
	Thermoregulation	
	Orientation	
	Chemoreception	
	Feeding	
	Predator evasion	
	Aggression/Territoriality	
	Learning depression	
	Growth	
	Disease resistance	
	Reproductive capacity	
	Survival	

1.3.2 Parasitic Infections

Although numerous parasites are known to infect wild (Munday *et al.* 2003) and ranched SBT (see Nowak *et al.* 2006), only four are believed to cause adverse affects on SBT health: *Uronema nigricans*, *Caligus chiastos*, *Hexostoma thynni*, and *Cardicola forsteri*.

Uronema nigricans is a free living ciliate that can infect SBT brain and cause encephalitis, the condition is called 'swimmer syndrome' due to the unusual behavior of infected fish (Munday *et al.* 2003). In the past infections have been linked to mortalities (Munday *et al.* 2003), however the threat to ranched SBT has been largely controlled due to the movement of ranching sites into deep water, with less suspended sediments and higher flush rates, reduced stocking densities, and improved feeding practices (Deveney *et al.* 2005, Nowak *et al.* 2006).

Sealice, primarily *Caligus chiastos*, can be found mostly on the head and eyes of bluefin tuna. Large infections were noted in the late 2000s, correlated with reduced condition (Hayward *et al.* 2008a) and eye damage (Hayward *et al.* 2008a, 2009). Within the ranching season, sealice infection commonly increases from 0% to 55% prevalence from transfer to week 6 of ranching, and then subsequently declines to zero over the next 3 months (Hayward *et al.* 2009). Unlike sealice infections of other fish, larval sealice are rarely detected on SBT (Hayward *et al.* 2010). Degen's leatherjacket, *Thamnaconus degeni*, a scavenger fish attracted to the tuna cages due to excess feed carries large numbers of *Caligus chiastos* chalimi and is the most likely source of SBT infection (Hayward *et al.* 2011). In recent years, the numbers of sealice on SBT have declined considerably, owed primarily to enhanced feed management.

There are three species of metazoan ectoparasites commonly found on the gills of ranched SBT: two copepods, *Pseudocycnus appendiculatus* and *Euryphorus brachypterus*, and a monogenean, *Hexostoma thynni* (see Nowak *et al.* 2006). While copepods are a problem in other aquaculture systems (Tully & Nolan 2002), due to their low prevalence, abundance (Hayward *et al.* 2007) and little to no host response, they are believed to pose little threat to SBT ranching (Deveney *et al.* 2005). The monogenean, *H. thynni*, does cause a significant host response in ranched SBT, including gross swelling at the site of attachment, therefore is of concern (Nowak *et al.* 2006). Although prevalent, the intensities of *H. thynni* remain low throughout ranching (Hayward *et al.* 2007, 2008b), do not proliferate and there is no

association between *H. thynni* infection and condition index (Hayward *et al.* 2007). Therefore, the existing infection is not believed to significantly affect production costs.

Finally, the blood fluke *Cardicola forsteri* has been identified as the most significant health risk to SBT today. *C. forsteri* has a two-host lifecycle, requiring a definitive host, bluefin tuna, and an intermediate host, a marine polychaete *Longicarpus modestus* (see Cribb *et al.* 2011) (Figure 1.1). While *C. forsteri*, are usually not present at transfer to sea cages, both prevalence and intensity increase during the first two months of ranching (Aiken *et al.* 2006). Presently, blood fluke infection in SBT has not been conclusively linked to mortality, but numerous pathological effects have been proposed (Cribb *et al.* 2000; Rough *et al* 2000; Colquittt 2001; Munday *et al.* 2003; Denis *et al.* 2011). Understanding the effects of and devising ways to reduce *C. forsteri* infection are currently a major priority for the Australian SBT ranching industry.

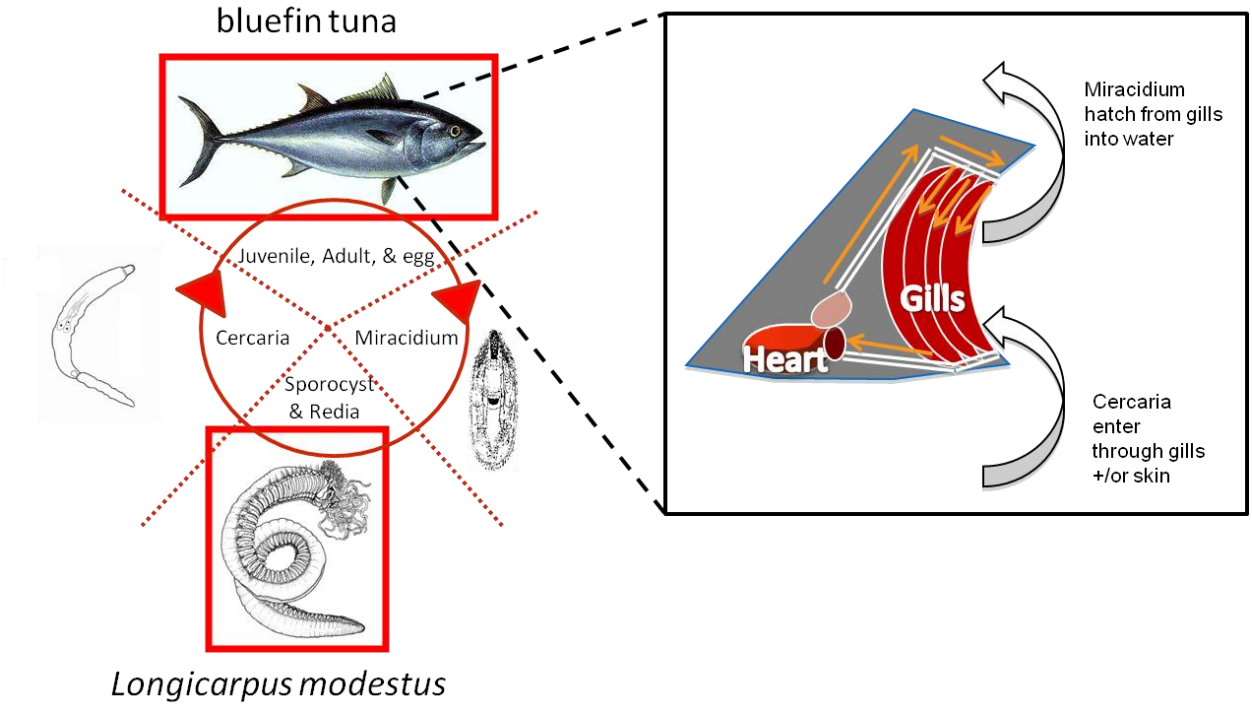

Figure 1.1 Lifecycle of *C. forsteri*. Illustrations of cercaria are from Cribb *et al.* (2011), miracidium are from Køie (1982) & Køie and Patersen (1988), and *Longicarpus modestus* are from Ross *et al.* (2000).

1.4 Research Objectives

- Determine whether the immune system of ranched SBT can be enhanced through dietary supplementation of vitamins and immunostimulants
- Describe the effects of ranching, specifically during the annual mortality event, on SBT health
- Investigate the effect of offshore ranching on SBT
- Describe the humoral immune response associated with the presence of life stages of *C. forsteri* within SBT
- Propose husbandry management strategies for reduction and elimination of *C. forsteri* infections in ranched SBT

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CHAPTER 2:

EFFECTS OF IMMUNOSTIMULANTS ON RANCHED SOUTHERN BLUEFIN TUNA *T. MACCOYII*: IMMUNE RESPONSE, HEALTH, AND PERFORMANCE

Kirchhoff, N.T., Antignana, T.D., Leef, M.J., Hayward, C., Wilkinson, R.J., Nowak, B.F., (2011). Effects of immunostimulant on ranched southern bluefin tuna *T. maccoyii*: immune response, health, and performance. Journal of Fish Biology 79: 331-355.

Abstract

Ranched southern bluefin tuna, *Thunnus maccoyii*, were fed baitfish supplemented with vitamins (predominantly E and C) or vitamins and immunostimulants, nucleotides and β-glucans, over 12 weeks after transfer and monitored for enhancement in immune response, health and performance through their 19 week grow-out period. Fish from two different tows were sampled separately at three different sampling points: at transfer to grow-out pontoons, at 8 weeks post transfer, and at harvest, 19 weeks post transfer. Lysozyme activity was enhanced during vitamin supplementation compared to control fish. Performance (i.e. survival, condition index, and crude fat), health (i.e. blood plasma variables including pH, osmolality, cortisol, lactate, and glucose), and alternative complement activity were not commonly improved through diet supplementation. There were some tow specific improvements in performance through vitamin supplementation including survival, selected parasite prevalence and intensity, and alternative complement activity. Immunostimulant supplementation also showed a tow specific improvement in plasma cortisol level. Tow specific responses may suggest that life history, previous health condition, and husbandry can affect the success of vitamin and immunostimulant enhancement of immune response, health, and performance of ranched southern bluefin tuna.

2.1 Introduction

The ranching of southern bluefin tuna, *Thunnus maccoyii* (Castelnau 1872), based in Port Lincoln, South Australia, began in 1991 and has since become one of Australia's most successful aquaculture industries; marketed in Japan, the United States of America, and Europe for high-end sashimi. In 2009, the Australian industry produced 7763t of *T. maccoyii* (gilled and gutted), worth AUD$120 million (Australian Southern Bluefin Tuna Industry Association (ASBTIA), pers. comm.). *Thunnus maccoyii* are captured in the Great Australian Bight at 2-4 years of age and are towed to near shore grow-out cages for a 2-8 month period of fattening on baitfish (ASBTIA, pers. comm.). A tow is a group of fish which was towed in the same tow cage from the fishing grounds to the ranching area. Each company may ranch fish from a number of tows which may arrive to the ranching area at different times. Through the capture, tow, transfer, and fattening operation, *T. maccoyii* are subjected to various kinds of stressors and are exposed to potentially pathogenic organisms (Munday *et al.* 2003, Nowak 2004, Deveney *et al*. 2005, Mladineo *et al*. 2008). When stress, which may lead to immunosupression (Snieszko 1974, Pickering & Pottinger 1989, Barton & Iwama 1991), is coupled with pathogen exposure, mortality and reduced performance may occur. The feeding of *T. maccoyii* with baitfish, which are variable and often deficient in nutritional content, could also be contributing to immunosuppression in ranched fish. Therefore, economic performance of ranched *T. maccoyii* could be enhanced if reduced stress or elevated immunocompetency can be achieved.

There are two ways to increase fishes' immunocompetence: vaccines and dietary immunostimulation. Vaccinations may be the most effective prophylactic method of controlling disease in fishes (Sakai 1999), but they are limited to only a small range of pathogens. Commercial vaccines available in aquaculture are only effective against bacterial and some viral diseases (Leong & Fryer 1993), but there are no commercial vaccines within the literature against parasitic diseases. Furthermore, an immediate control of an unknown disease agent is not possible through vaccination (Sakai 1999). Unlike vaccines, immunostimulants can enhance the overall resistance of an animal and are often made from naturally occurring dietary components. The potential benefits of using a prophylactic immunostimulant are many: reducing mortality, preventing diseases by enhancing overall disease resistance, enhancing the efficiency of anti-microbial immune pathways, increasing immunocompetence during sub-optimal environmental

conditions and enhancing resistance to parasites (Raa 2001). Use of immunostimulants has also been linked to increased growth and better feed conversion ratios (Sakai 1999). There are over 46 different types of immunostimulant products identified today, organized into seven categories: chemical, biological polysaccharides, animal and plant extracts, vitamins, hormones/cytokines, and nucleotides (Sakai 1999).

The immunostimulants used in this trial were chosen because of their solubility in water, natural occurrence, availability of background information, broad effects, and relatively low cost. Vitamin C (ascorbic acid) and E (α-tocopherol) are among the most studied compounds related to disease resistance in fishes (Raa 2000). Most fishes lack the ability to make sufficient vitamin C for their metabolic needs, therefore it must be provided in the diet (Dabrowski 2001). Natural food organisms such as plankton are rich in Vitamin C (Robinson & Li 1996), yet it was suggested that baitfish, which are fed to commercially ranched *T. maccoyii*, can be deficient in antioxidants such as vitamin C (Munday *et al*. 1997). Vitamin C inactivates oxygen radicals to protect cells from injury and promotes the oxidative denaturing of bacteria (Roth & Kaeberle 1985). Vitamin E increases resistance to disease by promoting antibody production through B-cell proliferation and reduces cell damage through phagocyte activation, as shown in mice (Galeotti 1998). Together, Vitamin C and E have the ability to stimulate antibodies (Navarre & Halver 1989), and to decrease the release of cortisol after an acute stressor (Li & Robinson 1999).

The immunostimulatory potential of β-glucans was first discovered in the 1960s, through their effect on tumor suppression in mice. Since then β-glucans have proven effective against bacterial, fungal, viral and protozoan pathogens (DiLuzio *et al*. 1985). β-glucans are components of the cell walls of mushroom and yeast; a particulate carbohydrate containing glucose and mannose (Dalmo & Bogwald 2008, Raa 1996). β-glucan has been demonstrated to enhance wound healing, repair of damaged cells (Raa 2000), and increase growth rate in several fishes (Ai *et al.* 2007, Cook *et al*. 2003, Misra *et al*. 2006, Sealey *et al*. 2008). In addition, β-glucans have been found effective in enhancing resistance against parasites. Atlantic salmon *Salmo salar* L. showed a 37.8% reduction in the mean number of sea lice, *Lepeophtheirus salmonis*, per fish when given a diet supplemented in nucleotides and β -glucans (Burrells *et al.* 2001a). The stimulating effect varies with the composition of the glucan, route of administration,

dosage, timing of feeding, species of fishes, fishes age, combination with environmental pathogens, and environmental temperature (Ainsworth *et al*. 1994, Dalmo & Bogwald 2008).

Nucleotides are the structural subunits of RNA and DNA, present in all living and non-living material. Two explanations have been suggested as to why fishes require dietary supplementation of nucleotides during stressful events: (1) commercial fishes feeds are currently formulated with an adequate supply of exogeneous nucleotides when under normal, non-stressful conditions (Burrells *et al*. 2001b, Low *et al*. 2003); (2) nucleotides in their free form are stable and difficult to digest therefore a cocktail of free nucleotides is required to overcome stressful conditions (Borda *et al*. 2003). Digestion and absorption competency of exogenous nucleotides is species specific, for example humans digest nucleotide intermediates while mice digest nucleosides (Sonoda & Tatibana 1978). Research on fishes is limited mostly because nuclease, the most important enzyme for nucleotide digestion is poorly understood, although present in rainbow trout *Oncorhynchus mykiss* (Walbaum 1972) (see Roald 1978). Dietary enhancement of nucleotides has caused increased growth in larval fishes (Li and Gatlin 2006) and reduced stress in several species fishes (Burrells *et al*. 2001b, Low *et al*. 2003) together with reducing the immune-inhibiting effects of cortisol (Leonardi *et al*. 2003), thereby increasing disease resistance against viral, bacterial, and parasitic pathogens (Li & Gatlin 2006).

The aim of this research was to enhance the immune response, health, and performance of ranched *T. maccoyii* through dietary supplementation. A broad application of the most commonly used immunostimulants, vitamin C (ascorbic acid), vitamin E (α-tocopherol), β-glucans, and nucleotides, was applied to the diet for twelve weeks post transfer into sea pontoons, and fish were monitored until harvest, after nineteen weeks of ranching. It was hoped that supplementation would help overcome the nutritional variability of the baitfish themselves and potential inhibitory effects of environmental stressors on *T. maccoyii* through the ranching period.

2.2 Materials and Methods

2.2.1 Experimental Fish and Study Design

Two assemblages of approximately 10000 wild *T. maccoyii* were captured by purse seine in the Great Australian Bight on 19 March 2009 and 23 March 2009, respectively. Following transport to the Tuna Offshore Farming Zone (TOFZ) in a towing pontoon, each towing pontoon was transferred into three grow-out pontoons on 28 March 2009 and 31 March 2009, respectively, and identified as tow 1 and tow 2 in order of arrival to the TOFZ. A total of 6 pontoons: two 'Control', two 'Vitamin', and two 'Immunostimulant' were used for the experiment, with each distinct tow containing one pontoon per treatment. For 12 weeks, the *T. maccoyii* in the grow-out pontoons received the standard commercial baitfish diet ('Control'), standard commercial baitfish supplemented with a multi-vitamin premix, formulated by D'Antignana and produced by DSM Nutritional Products (www.dsm.com, product #YG09473001) containing primarily vitamins C (ascorbic acid) and E (α tocopherol acetate) ('Vitamin'), and standard commercial baitfish supplemented with multivitamin premix with the addition of nucleotide (Optimun©) and ß-glucan (Sanictum©) from Chemoforma Ltd. (www.chemoforma.com) ('Immunostimulant'). The dietary supplements within a treatment were mixed and injected into the baitfish with an industrial meat marination injector (Convenience Food Systems *Accujector 450*, www.cfs.com), plate frozen into ~20kg blocks and stored at -20^{o}C until feeding. The immunostimulants were shown to be stable in frozen baitfish (D'Antignana, unpublished). The bait injection rate was calculated to deliver the following dose of nutrients per kg of baitfish: Ascorbic acid = ~2000mg, α tocopherol acetate = ~2400mg, *Optimun*$^{©}$ = ~4000mg and *Sanictum*$^{©}$ = 5.2mg. *T. maccoyii* were then fed the treatment diets every second day at ~ 35% of their daily feed ration for approximately 12 weeks, at a feed rate of approximately 1.14kg fish^{-1} day^{-1} in all grow-out pontoons. Common *T. maccoyi* parasites including Copepoda, Monogenea and Digenea are killed by freezing so there is no risk of infection resulting from feeding *T. maccoyi* frozen baitfish (B. Jones, pers. comm.). The standard commercial baitfish diet is a mix of domestic and imported small prey fishes.

2.2.2 Sample Collection: Field Collection

Thunnus maccoyii samples were taken at three different time points throughout the trial: initial (at the transfer to grow-out pontoons), 8 weeks post transfer and at a commercial harvest (19 weeks post transfer). Initial samples were taken from *T. maccoyii* between 90 and 101cm fork length at transfer, 20 fish per tow, 40 fish total. After 8 weeks of diet inclusion and at harvest, 20 fish were sampled from each pontoon. At the initial and 8 week time points, *T. maccoyii* were selected using a baited hook-and-line. Divers caught the *T. maccoyii* at harvest during the typical commercial harvest. The total time between capture and killing of fish was <1min for both collection methods.

Length and mass was recorded for all fish at the time of sampling, prior to gilling and gutting. Condition index was calculated for each fish using the formula: mass (kg) / [length (m) ^3]. Cerebrospinal fluid was obtained from the cranial orifice left exposed by the 'Taniguchi tool' using a transfer pipette. External metazoan parasites were counted from both the skin and gill arches by the naked eye. All lice visible to the naked eye were collected as soon as possible; any additional lice remaining on fish surfaces were then detected using a technique described in Hayward *et al.* (2010). All lice *Caligus* spp. were collected and preserved in ethanol, and identified later in the laboratory using a dissecting microscope. Immediately after external surface examination, whole blood was collected from the severed pectoral artery behind the pectoral recess in 2-9ml Vacutainer ® tubes (BD, www.bd.com), one heparinized and one non-heparinized, and placed on ice. Blood was collected within 3min of fish capture. The heart was placed in a waterproof tub and also stored on ice. One olfactory rosette was dissected from each fish and preserved in a jar with 10% neutral buffered formalin for further histological analysis of the presence of *Uronema nigricans* (Florentin). For vitamin and fat determination, tail muscle samples were collected from 20 fish at the initial time point and from 14 fish per pontoon at 8 weeks post transfer and at harvest and stored on ice.

2.2.3 Sample Collection: Laboratory Processing

The heparinized vial of whole blood was used for whole blood and plasma aliquots. Three 500ml aliquots of whole blood was transferred into 1.5ml plastic tubes and frozen at -80°C. The remaining blood was centrifuged at 3000xg at 4°C for 5min. Blood plasma was aliquoted into 5-1.5ml plastic tubes, and frozen

at -80°C. The non-heparinized vial of whole blood was used for serum collection. Vials were stored upright at 4°C for 24h, centrifuged at 1000xg at 4°C for 5min, and serum aliquoted into 3-1.5ml tubes. Serum samples were stored at -80°C. No serum was collected from tow 2 fish at transfer. Hearts were dissected open 2 to 4h after removal from the carcass and flushed with physiological saline to dislodge any adult blood flukes, *Cardicola forsteri* (Cribb) (see Aiken *et al*. 2006). Flushes were then poured into Petri dishes and examined for the presence of *C. forsteri* using a dissecting microscope. *T. maccoyii* cerebral fluid and olfactory rosettes were examined for the presence of *U. nigricans* following the method of Deveney *et al.* (2005).

T. maccoyii muscle tail cuts were individually homogenised in the laboratory within several hours of harvest then placed immediately in -80^{o}C storage until analyzed for vitamins C, E and crude fat.

2.2.4 Blood Parameters: Haematology

Hemaoglobin concentrations were determined from whole blood aliquots using the cyanometahemaoglobin assay based on Brown (1984). Briefly, samples were thawed then mixed for 10s using a vortex mixer. Blood was diluted 1:50 with Drabkin's reagent (Sigma-Aldrich, www.sigmaaldrich.com) (DR) and left to stand protected from light for 15 min. Hemaoglobin standards were set by preparing a 1:100 stock solution of dried hemaoglobin from bovine blood (Sigma-Aldrich, USA) in DR and then performing dilutions to obtain, 0, 5, 10, 15, 20, 25 and 50g dl^{-1} standards. Standards and samples were vortexed again for 10 s and 40 µl of each were placed in triplicate wells of a 96-well microtiter plate along with 160 µl of DR. Their absorbance was measured using a Rainbow Thermo plate reader (Tecan Trading AG, www.tecan.com) at a wavelength of 540nm, shaking the plates for 120s before reading. Hemaoglobin values <6.8 g dl^{-1} were assumed to be due to collection error (i.e. water contamination or failure to invert tube prior to aliquot collection) and were omitted from the dataset. This value was chosen based on the lower limits for *T. maccoyii* found by McGowan (2006) and Valdenegro (2009).

Blood plasma glucose and lactate were measured using a GM7 Microstats reader (Analox instruments, www.analox.com). The pH of blood plasma samples was measured using a Minilab Isfet pH meter Model

IQ125 (IQ Scientific, www.iqscientific.com). Blood plasma osmolality was obtained using a Vapro© Model 5520 vapour pressure osmometer (Wescor Inc., www.wescor.com).

Blood plasma cortisol was measured using a tritiated radioimmunoassay following standard ethyl acetate extraction of samples as previously described (Pankhurst and Sharples 1992).

2.2.5 Blood Parameters: Humoral Immune Response

Blood serum was analyzed in triplicate for lysozyme activity, and alternative complement activity. No serum was collected from tow 2 at transfer; therefore no data are available for humoral immune response for this time point. Lysozyme activity was measured using a method based on that described by Carrington and Secombes (2007). Briefly, serum samples were diluted 1:20 in a phosphate/citrate buffer (0.1 M) and compared to a series of standards made from hen egg white lysozyme (HEWL) (80, 40, 20, 10, 5 and 0 µg ml^{-1}). In a flat-bottom, 96-well plate, 25 µL of each standard and each serum sample were pipetted in triplicate. Using the same buffer, a 1mg ml^{-1} suspension of *Micrococcus luteus* (Schroeter) was prepared and 175 µl of the bacterial suspension was added to each well using a multichannel pipette. The plate was shaken and the reduction in turbidity was immediately measured in a Rainbow Thermo plate reader (Tecan Trading AG, www.tecan.com) at 450 nm over 11-30 s intervals in negative kinetics mode at 25^{o}C. Lysozyme activity values were only accepted if they fell between 10 and 40 µg ml^{-1}. Samples that fell outside this range were diluted at 1:10 or 1:40, respectively.

Blood serum alternative complement activity was measured using a modified Yano (1992) method. Briefly, sheep red blood cells (SRBC) were rinsed and concentration adjusted for optimal absorbance. *Thunnus maccoyii* serum was diluted 1:40 in chelated HBSS and 0.2, 0.25, 0.3, 0.35 & 0.4 ml of each sample was made up to 1 ml in chelated HBSS. A 200 µl aliquot of each sample dilution was placed in triplicate in a v-bottom 96-well plate along with a 0% and 100% lysis standard (chelated HBSS and distilled water, respectively). Using a multichannel pipette, 80 µl of SRBC was added to each well. The plate was then incubated at 25°C for 90min. Each plate was centrifuged at 1000xg at 4°C for 5 min with no break and 150 µl supernatant from each well transferred to a flat-bottom 96-well plate. Absorbance was read at 414 nm. Only values of y between 0.35 and 0.7 were used for calculation. Samples falling outside this range were repeated at a different dilution.

2.2.6 Vitamin and fat content of muscle

Muscle crude fat was determined by an adapted version of the Norwegian Standard method (NS 9402 E). Fat was extracted from homogenised muscle tissue in ethyl acetate and sodium sulphate in a stomacher mixer (IUL Instruments, www.iul-inst.com) for several minutes. The mixture was allowed to settle then the clear upper layer of solvent was decanted through GF/C filter paper. The solvent was then measured in to pre-weighed beakers, evaporated overnight under a fume hood, then placed in a drying oven at ~70^0C for 1h to ensure all moisture was evaporated. Crude fat was then determined gravimetrically by weighing the beakers.

Vitamin C was determined by a technique based on the HPLC fluorometric method (Brown and Miller 1992), which was adapted to *T. maccoyii* muscle at the Lincoln Marine Science Centre with the assistance from Malcolm Brown (CSIRO Hobart Tasmania). Briefly, muscle tissue was homogenized in ice cold metaphosphoric acid / EGTA solution using an Omni Prep multi-homogenizer. The resulting mixture was centrifuged at 2147 xg then ascorbate oxidase (Roche Diagnostics, www.roche-australia.com) was added to the supernatant to reduce the extracted ascorbic acid to de-hydro ascorbic acid. 0.1% O-phenylene Diamine was added to induce florescence of the de-hydro ascorbic acid. Filtered extract was transferred to HPLC vial. Vitamin C was eluted by HPLC in a di-potassium phosphate / methanol mobile phase using an Grace Altima C_{18} 5 µm column, 250 mm x 4.6 mm (Crawford Scientific, www.crawfordscientific.com), Waters 2695 separations module and a Waters 2475 Fluorescence detector (355nm excitation and 425nm emission).

The vitamin E content of the muscle was determined using a HPLC fluorometric assay modified for use on *T. maccoyii* flesh from the method originally developed by Huo *et al*. (1999). Briefly, vitamin E was extracted by homogenizing in HPLC grade methanol / BHT using the Omni Prep Multi-homogenizer and then holding on ice for 3 h. The mixture was centrifuged at 2147 xg and filtered supernatant transferred to HPLC vial. Vitamin E was eluted by HPLC in a 98% methanol / 2% water mobile phase using a Grace Prevail C_{18} 5 µm column, 150 mm x 4.6 mm (Crawford Scientific), Waters 2695 separations module and a Waters 2475 fluorescence detector (296 nm excitation and 340 nm emission).

2.2.7 Statistics

Parasite infections were characterised, for each pontoon, by prevalence (the number of host infections as a proportion of the population at risk) and mean abundance (the average number of parasites in all hosts (Bush *et al*. 1997). Sterne's exact 95% confidence intervals were calculated for prevalence, and 95% bootstrap confidence intervals (with 2000 replications) were calculated for mean abundance, using the software 'Quantitative Parasitology 3.0' (Reiczigel and Rózsa 2005). The prevalence and mean abundance for each species for each pontoon were compared with other treatments and ranching durations in a pairwise fashion. Given the high total number of pair-wise comparisons, a significance level of $\alpha = 0.01$ was regarded as significant for these statistics. Plasma cortisol results were square root transformed for all statistical analyses due to their failure to pass the Bartlett test for normal distribution.

All blood and production results were interpreted using the R 2.8.1 statistical package (© 2008, The R Foundation for Statistical Computing, www.r-project.org). Effect of tow, treatment, and time in culture were determined for each immune variable using an orthogonal design ANOVA. The assumption of homogeneity of variances was checked by the residual plot and Bartlett test and variables transformed when necessary. The Tukey HSD post-hoc test was applied at a significance level of $\alpha=0.05$, to determine differences between the explanatory variables. Plasma pH and muscle α-tocopherol concentration was log 10 transformed, serum lysozyme activity was sine transformed for all statistical analysis, and muscle crude fat composition was square root transformed due to failure to pass the Bartlett test for normalcy.

2.3 Results

2.3.1 Ranching performance

Absolute mortality from transfer to 7 weeks remained relatively stable at 2 to 3% (10-20 fish) for all grow-out pontoons regardless of treatment or tow (Figure 2.1). At week 7, a tow and treatment effect occurred. Within tow 1, mortality spiked within the Control and Immunostimulant treatments from week 7 to 12 showing approximately 14% mortality (~140 fish). *T. maccoyii* from the Vitamin treatment also had a spike in mortality but this did not occur until weeks 9 to 11, resulting in only 7.5% overall mortality (75

fish). There was no effect of treatment on survival for tow 2, with *T. maccoyii* in all treatments experiencing a mortality peak from week 8 to 11, resulting in between 8 to 9% overall mortality (80-90 fish). Post week 12, mortalities returned to background 2-3% level for both tows and all treatments.

There was no effect of treatment on the condition index (F= 1.5966, df= 2, 190, p=0.205) and there was no interaction between tow and treatment (F=0.9449, df= 2,190, p=0.39) (Table 2.1).

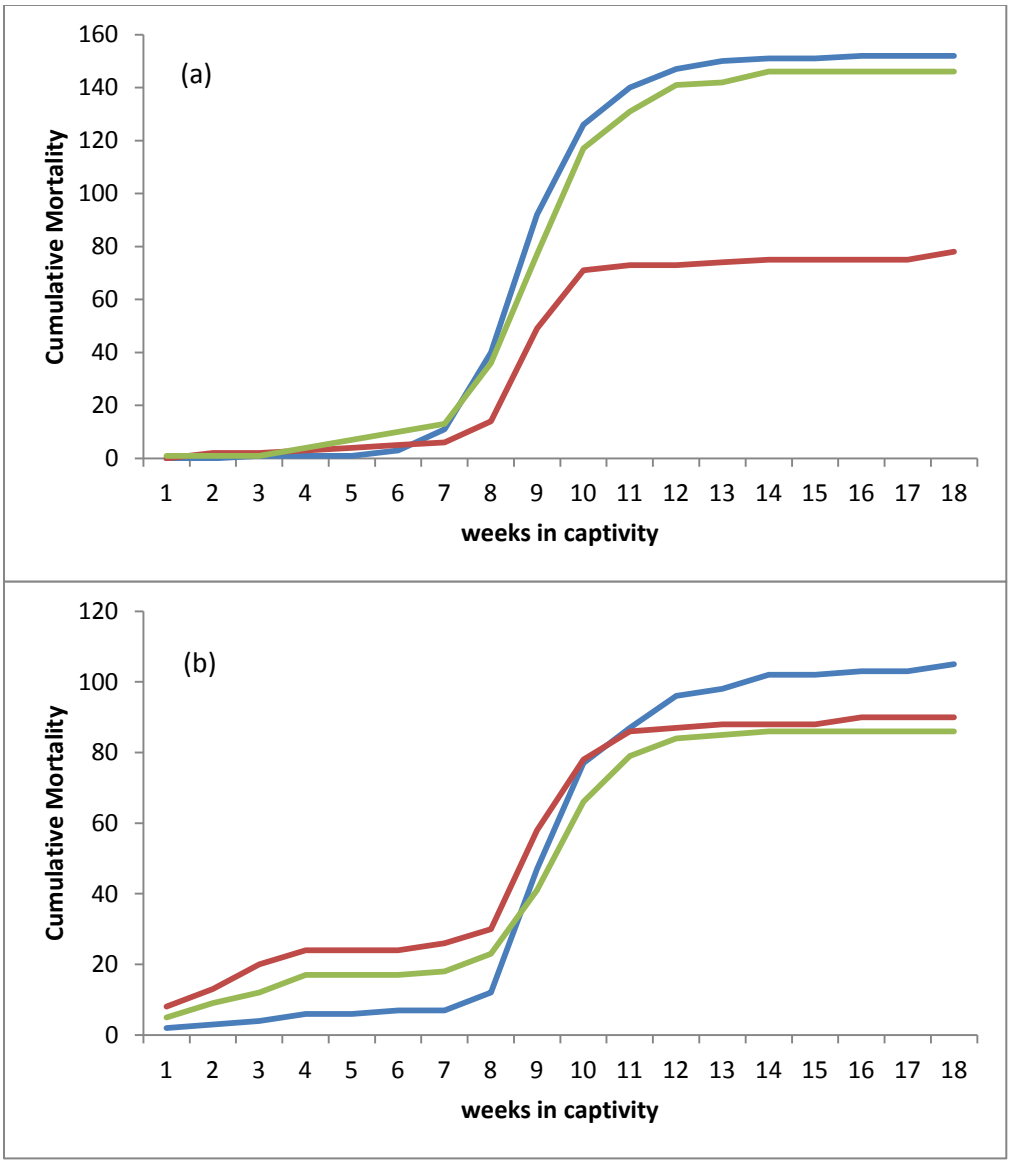

Figure 2.1 Cumulative mortalities from transfer into the sea cages until harvest for each treatment: Control (__), Vitamin (__) and Immunostimulant (__) for tows (a) 1 and (b) 2.

Table 2.1 Condition index, crude fat content, hemaoglobin (Hb) concentration, pH, and osmolality of *T. maccoyii* peripheral blood. Values are presented as mean ± se (n=7 at initial, n=20 at 8 weeks and harvest). Different superscript lower case letter denote statistical differences (p<0.01).

	Tow 1			Tow 2		
	Control	Vitamin	Immunostimulant.	Control	Vitamin	Immunostimulant.
Condition Index						
Initial	18.9±0.4	18.6±0.2	18.5±0.4	20.1±0.4	19.2±0.6	19.4±0.3
8 weeks	24.2±0.4	24.4±0.3	23.9±0.3	24.6±0.3	24.1±0.6	25.1±0.2
Harvest	24.4±0.3	24.1±0.3	24.3±0.4	25.3±0.5	24.6±0.3	24.7±0.4
Crude Fat (%)						
Initial	0.04±0.01	0.04±0.01	0.04±0.01	0.12±0.00	0.12±0.00	0.12±0.00
8 weeks	5.81±0.56	5.30±0.01	5.21±0.55	8.39±1.10	7.97±0.63	9.08±1.23
Harvest	5.40±0.53	5.28±0.47	5.77±0.42	5.94±0.44	6.54±0.47	7.76±0.52
Hb (g/dL)						
Initial	21.19±1.04	22.49±1.42	21.20±2.01	20.21±1.18	19.90±1.06	22.70±0.66
8 weeks	18.11±0.88	16.51±0.62	17.35±0.65	22.07±1.12	19.57±0.55	19.17±0.43
Harvest	23.27±1.47[a]	16.22±1.12[b]	18.45±1.32[b]	23.36±1.73[b]	19.97±1.25[b]	28.45±1.54[a]
pH						
Initial	8.24±0.04	8.25±0.04	8.28±0.02	8.24±0.02	8.20±0.04	8.20±0.05
8 weeks	7.94±0.02	7.97±0.03	8.00±0.02	7.98±0.03	7.99±0.02	8.00±0.02
Harvest	7.86±0.04	7.42±0.04	7.85±0.03	7.69±0.04	7.86±0.05	7.82±0.05
Osmolality (mmol/kg)						
Initial	431.60±22.8	397.4±4.9	404.2±3.9	402.4±7.5	417.1±7.4	415.8±10.2
8 weeks	443.6±12.1	415.9±7.7	420.6±1.9	421.2±7.6	404.0±2.7	442.6±6.4
Harvest	458.9±4.8	471.8±7.4	453.0±5.1	458.9±4.8	488.5±6.6	463.4±5.5

2.3.2 Vitamin and fat content of muscle

From transfer to 8 weeks post-transfer, a 4 fold increase in muscle vitamin C concentration was observed in the Vitamin and Immunostimulant treatments (Figure 2.2). At harvest, 18 weeks post-transfer, muscle vitamin C concentration for the Vitamin and Immunostimulant treatments remained elevated from initial levels, but considerably reduced from 8 week levels. By that time there was no significant effect of treatment on muscle vitamin C concentrations. The control treatment maintained relatively constant muscle vitamin C concentration throughout the sampling time points. There was no difference between the Vitamin and Immunostimulant treatments at any sample time. An interaction between ranching duration and treatment on muscle vitamin C concentration was observed ($F= 14.0336$, $df= 4, 189$, $p<0.001$). In addition, an interaction between tow and treatment on muscle vitamin C concentration was evident ($F=1.9386$, $df=2,189$, $p= 0.1468$).

From transfer to 8 weeks post-transfer, there was an increase in vitamin E for the Vitamin and Immunostimulant treatments (Figure 2.3). The Vitamin and Immunostimulant treatments had 1.5 times the muscle vitamin E concentration compared to the Control treatment at 8 weeks post-transfer. From 8 weeks post-transfer to harvest, muscle vitamin E concentration increased in fish from all treatments. At harvest the Vitamin and Immunostimulant treatments had ~3 times the initial muscle vitamin E concentration. The concentration in the muscle of *T. maccoyii* from the Control treatment at harvest was significantly lower than the other two treatment groups, at ~1.5 times the initial concentration. There was no difference for muscle vitamin E concentration between the Vitamin and Immunostimulant treatments at any sample date. There was an effect of ranching duration and treatment on muscle vitamin E concentration ($F=11.2726$, $df= 4,189$, $p<0.001$). There was no interaction between tow and treatment on muscle vitamin E concentration ($F= 0.7790$, $df= 2,189$, $p=0.4603$).

There was no effect of treatment on muscle crude fat content ($F=2.6860$, $df=2, 189$, $p= 0.071$) and no interaction between tow and treatment ($F=0.9142$, $df=2,189$, $p=0.403$) (Table 2.1).

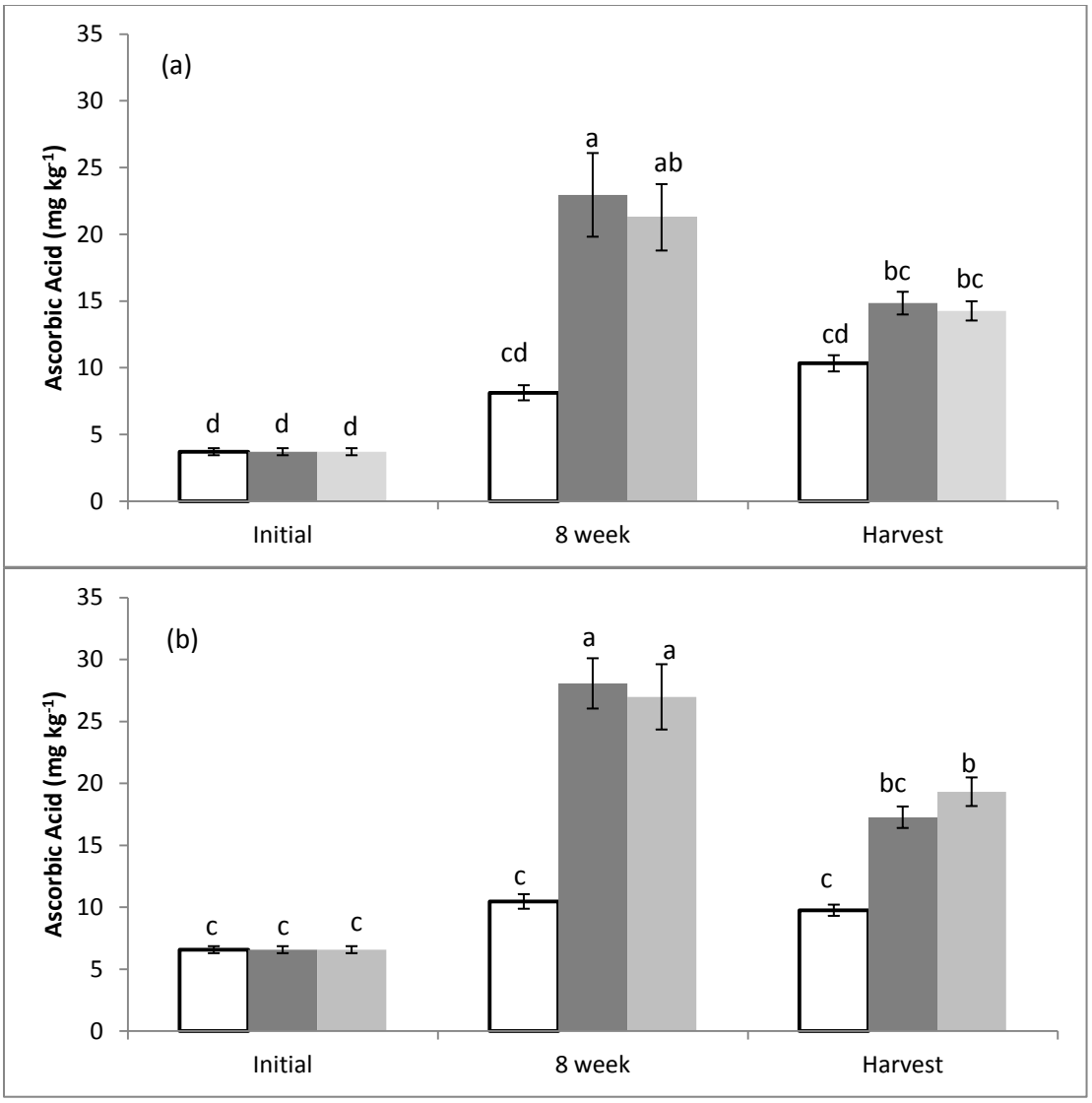

Figure 2.2 Vitamin C (ascorbic acid) concentration in tail muscle of *T. maccoyii* shown as mean ± S.E. for each treatment group: Control (□), Vitamin (■) and Immunostimulant (■) for tows (a) 1 and (b) 2. (n=7 at initial and n=20 for 8 weeks and harvest for each tow). Different letters denote significant differences at p<0.05.

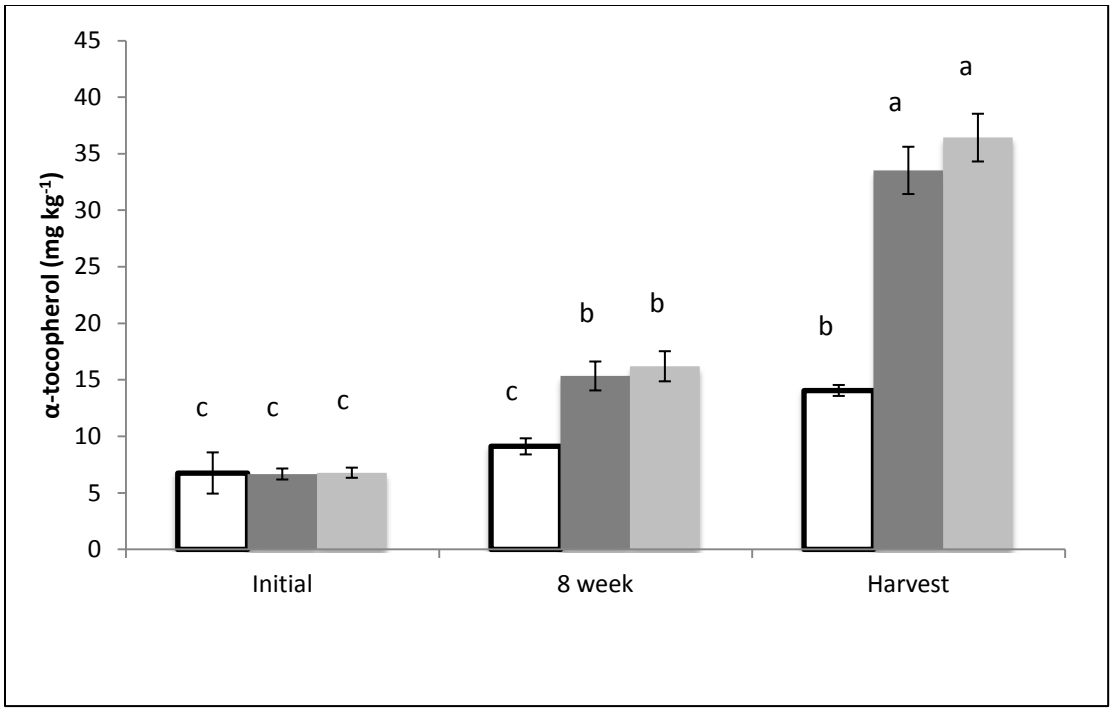

Figure 2.3 Vitamin E (α-tocopherol) concentration in tail muscle of *T. maccoyii* shown as mean ± S.E. for each treatment group: Control (□), Vitamin (■) and Immunostimulant (■). (n=14 at initial and n=40 for 8 weeks and harvest). Different letters denote significant differences at p<0.05.

2.3.3 Parasites

Six species of parasites were detected: on the skin and fins, two species of sea lice (*Caligus chiastos* Lin & Ho and *C. amblygenitalis* Pillai); on the gills, one species of monogenean (*Hexostoma thynni* Delaroche) and two species of copepods (*Pseudocycnus appendiculatus* Heller and *Euryphorus brachypterus* Gerstaecker); and in heart flushes, one species of blood fluke (*C. forsteri*). Given the relative rarity of *C. amblygenitalis* (five out of a total of 58 individuals, which were also all collected only at the time of harvest), results for both species of sea lice are pooled in figures and in statistical analyses. *Uronema nigricans* was not detected neither in the cerebrospinal fluid nor histological sections of olfactory rosettes in any fish sampled within this experiment.

Table 2.2 Comparison of mean prevalence and mean abundance of gill parasites recorded in this study. (n=7 at initial, n=20 at 8 weeks and harvest). n.a. denotes samples which were not available. No significant differences were found between treatments or ranching duration.

	Prevalence (%)			Mean Abundance		
	Control	**Vitamin**	**Immunostimulant**	**Control**	**Vitamin**	**Immunostimulant.**
Hexostoma						
tow 1						
Initial	n/a	n/a	n.a.	n.a.	n.a.	n.a.
8 weeks	15.00	15.00	25.00	0.45	0.45	0.40
Harvest	15.00	15.00	10.00	0.40	0.50	0.25
tow 2						
Initial	14.29	42.86	33.33	0.14	1.00	1.49
8 weeks	20.00	30.00	25.00	0.80	0.60	2.60
Harvest	15.00	10.00	15.00	0.75	0.40	0.25
Pseudocycnus						
tow 1						
Initial	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
8 weeks	5.00	5.00	10.00	0.30	0.40	0.25
Harvest	5.00	25.00	30.00	0.05	0.35	0.75
tow 2						
Initial	28.57	28.57	16.67	2.29	0.71	0.18
8 weeks	5.00	25.00	15.00	0.05	0.30	0.25
Harvest	40.00	20.00	5.00	0.80	0.85	0.20
Euryphorus						
tow 1						
Initial	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
8 weeks	10.00	10.00	5.00	0.40	0.15	0.05
Harvest	0.00	5.00	0.00	0	0.1	0
tow 2						
Initial	0.00	14.29	0.00	0	0.14	0.02
8 weeks	10.00	0.00	5.00	0.10	0.00	0.05
Harvest	0.00	0.00	5.00	0	0	0.05

For four of the five types of parasite monitored on and in tuna, there were no significant differences in prevalence for any date or for any treatments, in either of the two tows (*Caligus* spp.; *H. thynni*, *P. appendiculatus* and *E. brachypterus*) (Table 2.2). In contrast, for *C. forsteri*, in the first tow, prevalence in two of the groups (control and immunostimulant-fed *T. maccoyii*) was significantly higher at the end of the season than initially; for the vitamin treatment group in this tow, however, there was no significant change in prevalence over time (Figure 2.4). Thus, in this tow, at the end of the trial, prevalence was significantly lower in the vitamin-fed group than in the control and immunostimulant-fed *T. maccoyii*. For *C. forsteri* in

T. maccoyii in the second tow, however, prevalence was significantly higher among all three treatment groups at the end of the trial compared with the start; and there were no significant differences in prevalence among the three treatment groups at the end of the trial (Figure 2.4).

For three types of parasites monitored on and in *T. maccoyii*, there were no significant differences in mean abundance for any date or for any treatments, in either of the two tows: *H. thynni*, *P. appendiculatus* and *E. brachypterus* (Table 2.2). For the remaining two types of parasites, *Caligus* spp. and *C. forsteri*, there were similarly no significant differences between mean abundances in *T. maccoyii* sampled in the first tow, but there were some significant differences for both of these parasite types in the second tow (Figure 2.5; Figure 2.6). In the case of *Caligus* spp. in the second tow, mean abundance was significantly higher on control *T. maccoyii* at week 8 when compared with the initial mean abundance (Figure 2.5). In the case of *C. forsteri* in the second tow, mean abundance was significant higher on *T. maccoyii* fed immunostimulants at harvest time when compared with initial mean abundance (Figure 2.6).

2.3.4 Blood Analyses

Within tow 1, the *T. maccoyii* from Control group had an increased hemaoglobin concentration from 8 weeks post transfer to harvest, exceeding both the Vitamin and Immunostimulant cages, which remained constant (Table 2.1). Within tow 2, the Immunostimulant group increased in hemaoglobin concentration from 8 weeks to harvest, exceeding both the Control and Vitamin, which remained unchanged. There was no difference between treatments at harvest for either tow. There was an interaction between tow, treatment, and ranching duration on whole blood hemaoglobin (Hb) concentration ($F=3.8908$, $df=4, 251$, $p=0.004$).

For both tows, there was no difference in plasma glucose between initial and 8 week samples for any of the treatment groups (Figure 2.7). From 8 weeks to harvest, the fish from Control and Vitamin treatment groups increased in blood plasma glucose concentration while the glucose levels for Immunostimulant returned to initial values. There was an effect of interaction between treatment and ranching duration for plasma glucose concentration (mmol l^{-1}) ($F=6.4312$, $df=4,261$, $p<0.001$). There was no interaction between tow and treatment for plasma glucose ($F=0.9066$, $df=2,261$, $p=0.4052$).

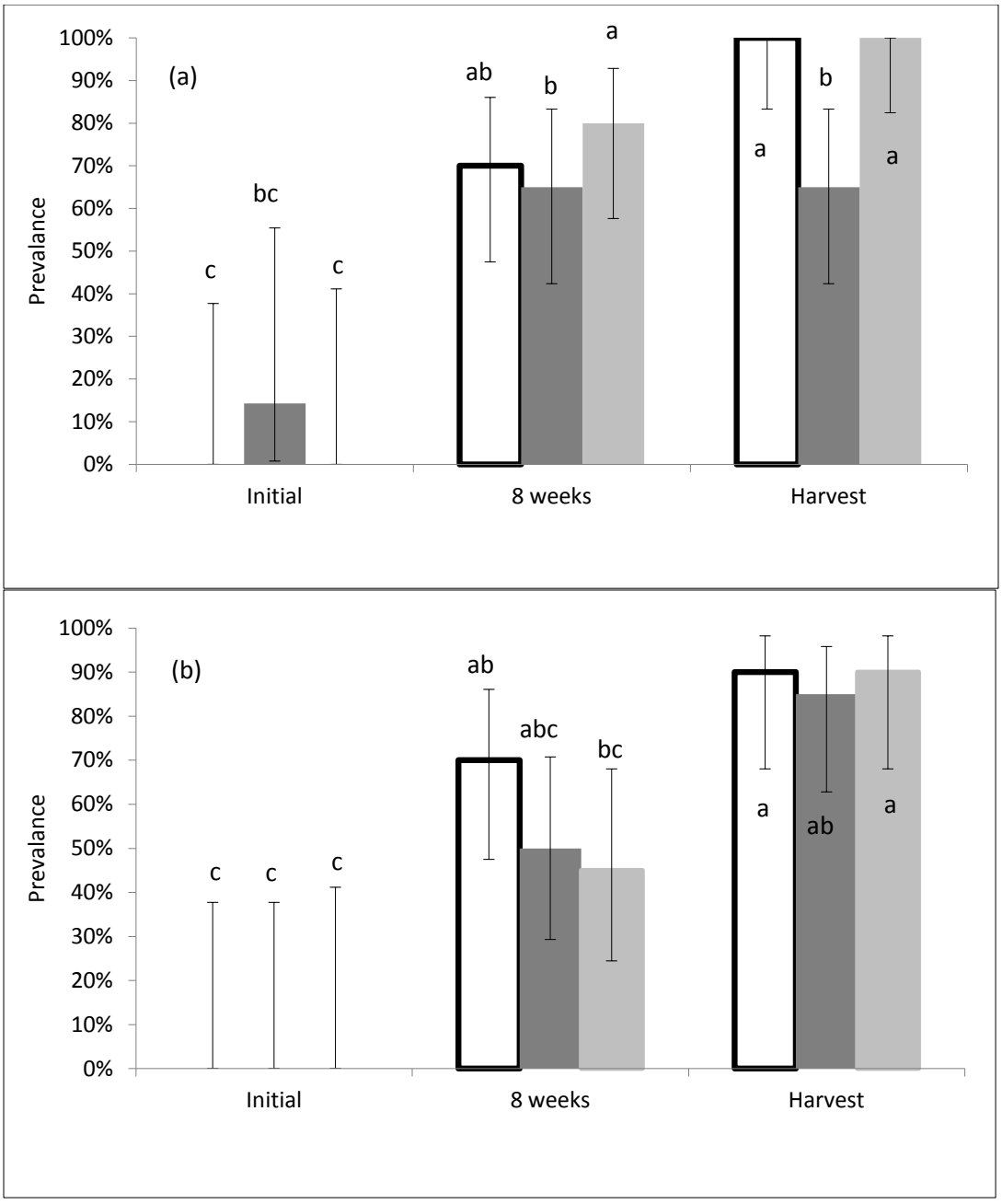

Figure 2.4 Effect of treatment and time on mean ± Sterne's exact 95% C.I. prevalence of *C.forsteri* for each treatment: Control (□), Vitamin (■) and Immunostimulant (■) for tows (a) 1 and (b) 2. (n=7 at initial and n=20 for 8 weeks and harvest). Different letters denote significant differences at p<0.05.

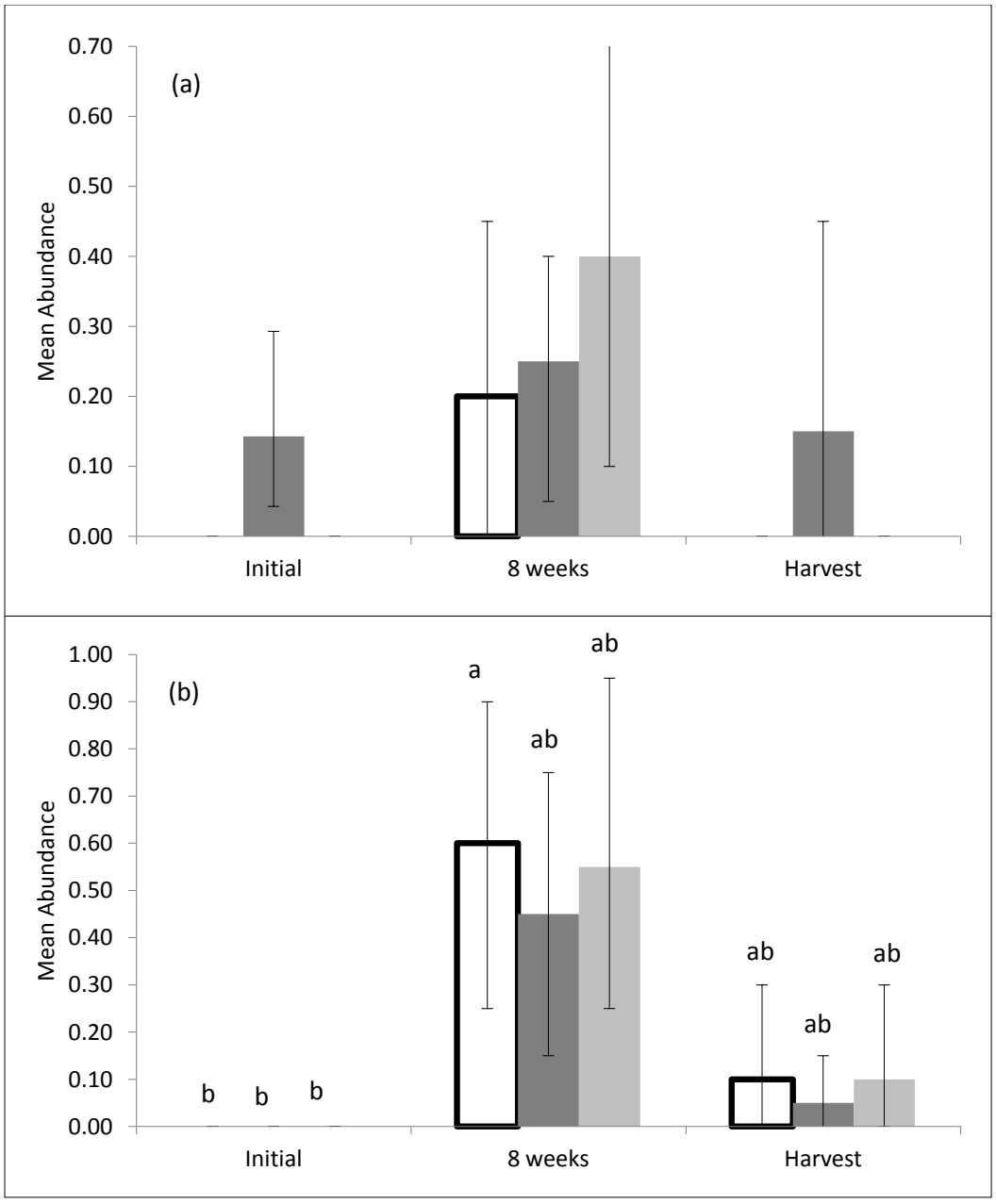

Figure 2.5 Effect of treatment and time on mean ± Sterne's exact 95% C.I. abundance of *Caligus* spp. for each treatment: Control (□), Vitamin (■) and Immunostimulant (■) for tows (a) 1 and (b) 2. (n=7 at initial and n=20 for 8 weeks and harvest). Different letters denote significant differences at p<0.05.

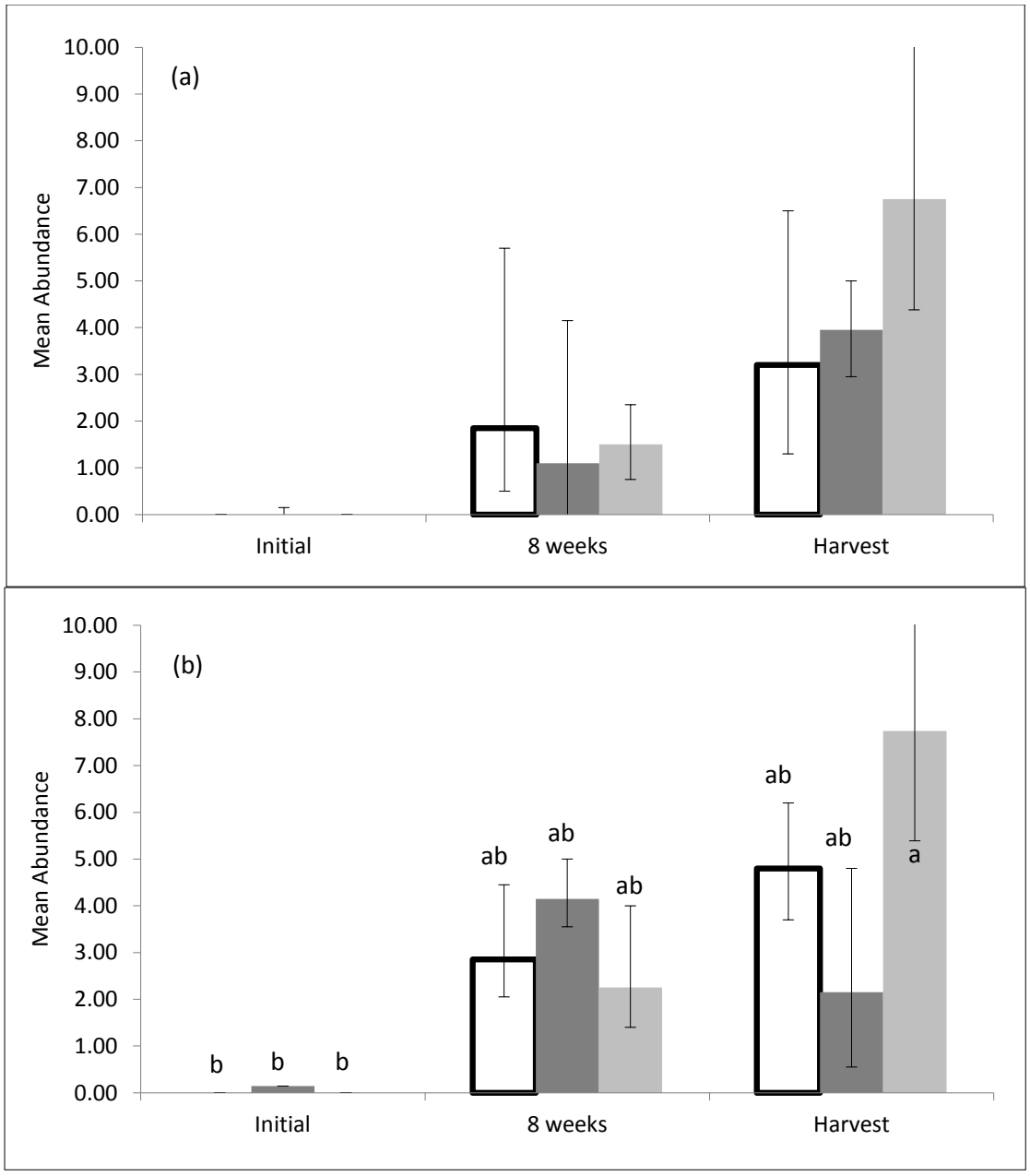

Figure 2.6 Effect of treatment and time on mean ± Sterne's exact 95% C.I. abundance of *C. forsteri* for each treatment: Control (□), Vitamin (■) and Immunostimulant (■) for tows (a) 1 and (b) 2. (n=7 at initial and n=20 for 8 weeks and harvest). Different letters denote significant differences at p<0.05.

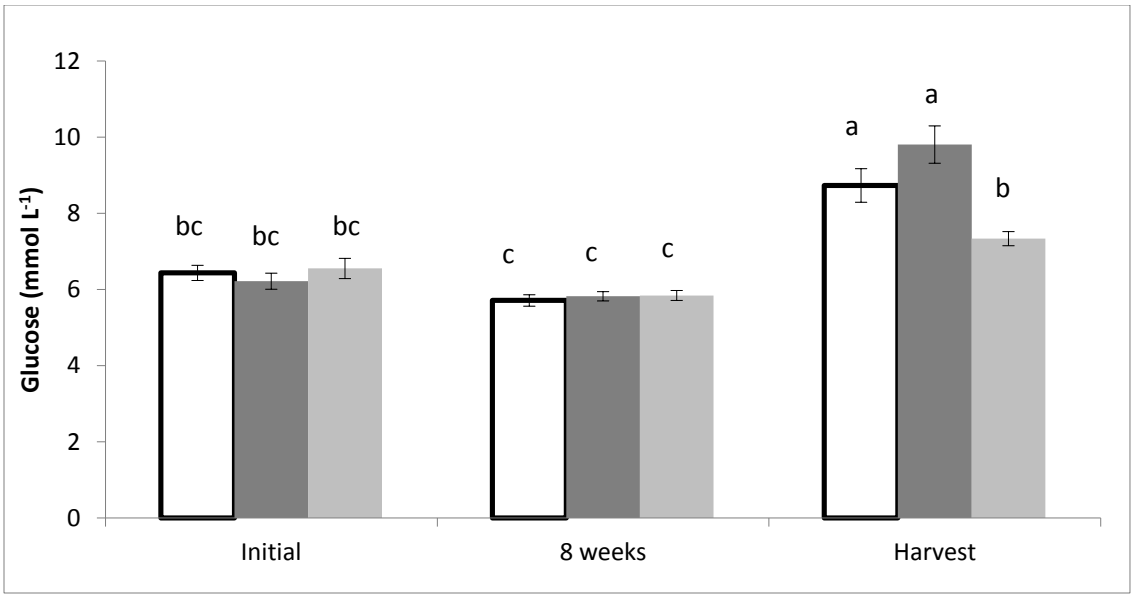

Figure 2.7 Plasma glucose from transfer to harvest, shown as mean ± S.E. for each treatment: Control (□), Vitamin (■) and Immunostimulant (■). (n=14 at initial and n=40 for 8 weeks and harvest). Different letters denote significant differences at p<0.05.

There was no effect of treatment on blood plasma lactate either initially or at 8 weeks (Figure 2.8). Fish from all treatments had elevated plasma lactate at harvest. At harvest, the fish from Vitamin treatment had higher plasma lactate than the Control or Immunostimulant treatments in tow 2. A similar trend occurred with Vitamin fish compared to Immunostimulant fish in tow 1. There was a significant interaction between tow and ranching duration for plasma lactate concentration ($F=3.2375$, $df=2,256$, $p=0.041$). There was also a significant interaction between treatment and ranching duration ($F=7.881$, $df=4,256$, $p<0.001$). There was no interaction between tow and treatment for plasma lactate concentration.

There was no treatment effect on plasma pH at any other time point for either tow (Table 2.I). There was an interaction between tow, treatment, and ranching duration for plasma pH ($F=13.9288$, $df=4,260$, $p<0.001$). This interaction was due to one pontoon, tow 1 Vitamin treatment, having extremely low average plasma pH at harvest, suggesting the occurrence of acidosis.

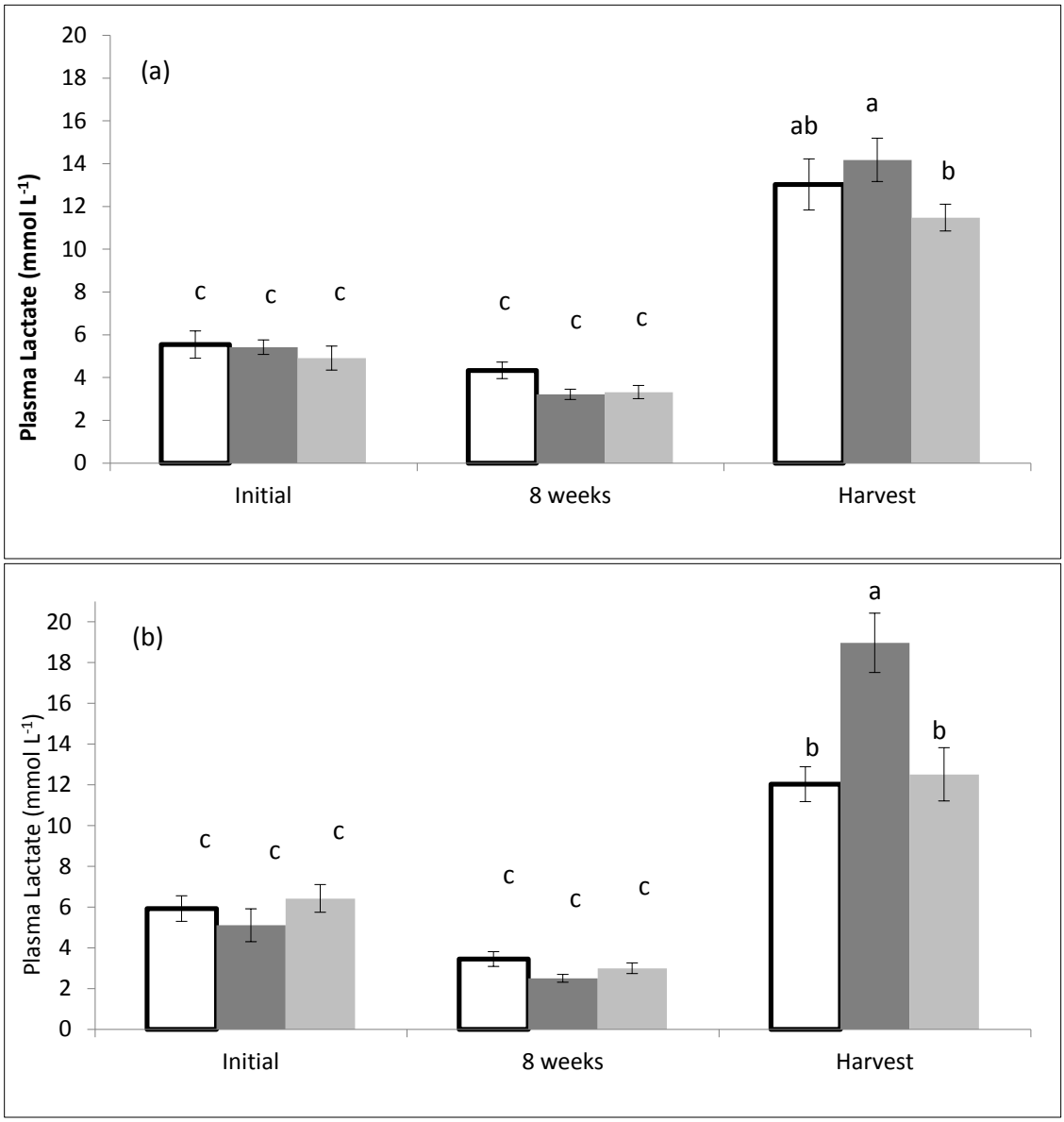

Figure 2.8 Plasma lactate from transfer to harvest shown as mean ± S.E. for each treatment: Control (□), Vitamin (■) and Immunostimulant (■) in (a) tow 1 and (b) tow 2. (n=7 at initial and n=20 for 8 weeks and harvest). Different letters denote significant differences at p<0.05.

Although there was an interaction between treatment and ranching duration (F=6.7292, df=4,260, p<0.001) and between tow and treatment on plasma osmolality (mmol kg^{-1}) (F=4.7222, df=2,260, p=0.01), there was no difference between blood plasma osmolality for fish from different treatments at any sampling point (Table 2.1).

For both tows, there was no change in plasma cortisol between initial and 8 weeks post transfer for any treatment (Figure 2.9). Within tow 1, there was no difference in plasma cortisol between treatments at any sampling time. Within tow 2, the Immunostimulant treatment had a significantly reduced cortisol concentration compared to the control treatment at 8 weeks post-transfer. With the exception of the Control treatment for tow 2, all other treatments had significantly higher plasma cortisol concentrations at harvest compared with 8 week samples. However, at harvest there was no difference between the Vitamin and Immunostimulant treatments and the Control treatment for either tow. There was no change in plasma cortisol for the Control treatment regardless of ranching duration for tow 2. There was an interaction between tow, treatment, and ranching duration for plasma cortisol ($F= 4.0013$, $df= 4, 260$, $p=0.004$).

2.3.5 Humoral Immune Response

The Vitamin and Immunostimulant treatments had ~1.5 times higher serum lysozyme activity compared to the Control treatment at 8 weeks (Figure 2.10). From 8 weeks to harvest, lysozyme activity decreased in *T. maccoyii* from all treatment groups, to a level not different from the initial samples. There was an interaction between treatment and ranching duration ($F=4.0142$, $df=4,245$, $p=0.004$), but no interaction between tow and treatment ($F=2.0954$, $df=2,245$, $p=0.125$) for serum lysozyme activity.

There was no treatment effect within tow 1 for alternative complement activity (Figure 2.11). Within tow 2, there was no difference between treatments at 8 weeks, but the alternative complement activity for Control treatment decreased at harvest, lower than both the Vitamin and Immunostimulant group. There was an interaction between treatment and ranching duration ($F=2.5370$, $df=4,242$, $p=0.04$) and between tow and treatment ($F=7.4262$, $df=2,242$, $p<0.001$) for serum alternative complement activity (units ml^{-1}), indicating a tow effect on treatment response.

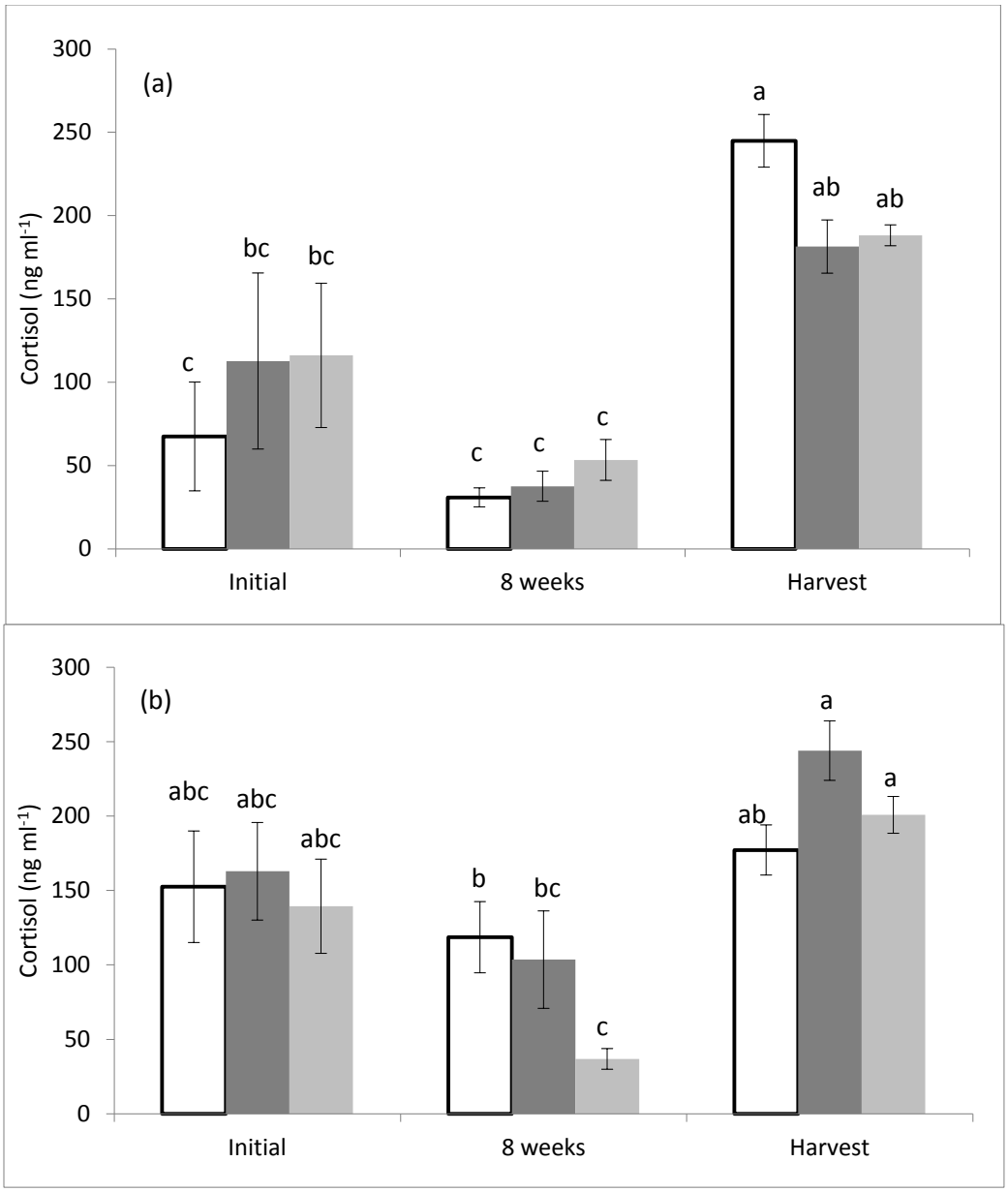

Figure 2.9 Plasma cortisol from transfer to harvest shown as mean ± S.E. for each treatment: Control (□), Vitamin (■) and Immunostimulant (■) for tows (a) 1 and (b) 2. (n=7 at initial and n=20 for 8 weeks and harvest). Different letters denote significant differences at p<0.05.

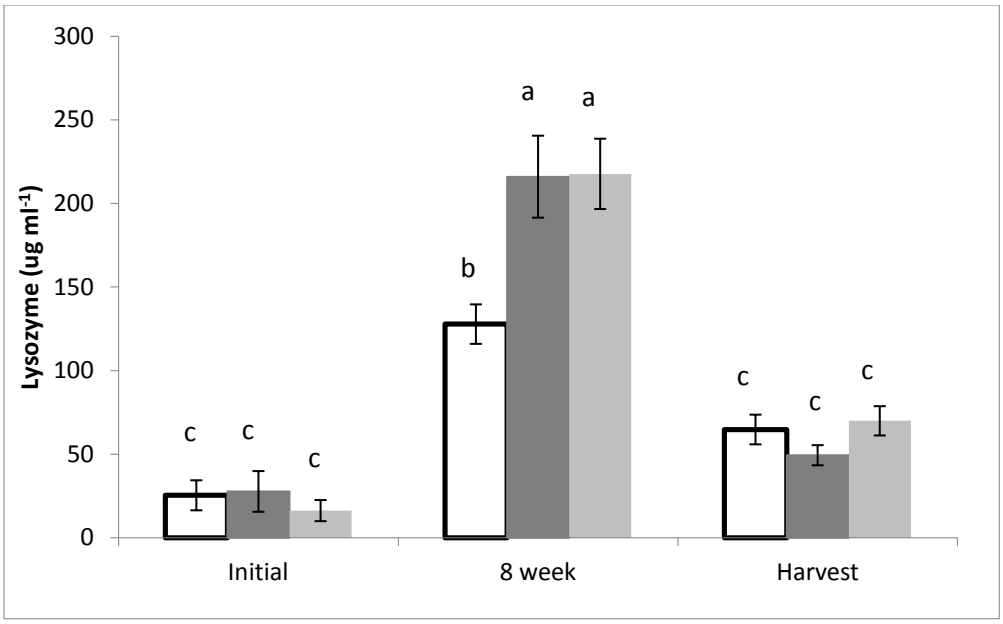

Figure 2.10 Lysozyme activity from transfer to harvest shown as mean ± S.E. for each treatment: Control (□), Vitamin (■) and Immunostimulant (■). (n=7 at initial and n=40 for 8 weeks and harvest). Different letters denote significant differences at p<0.05.

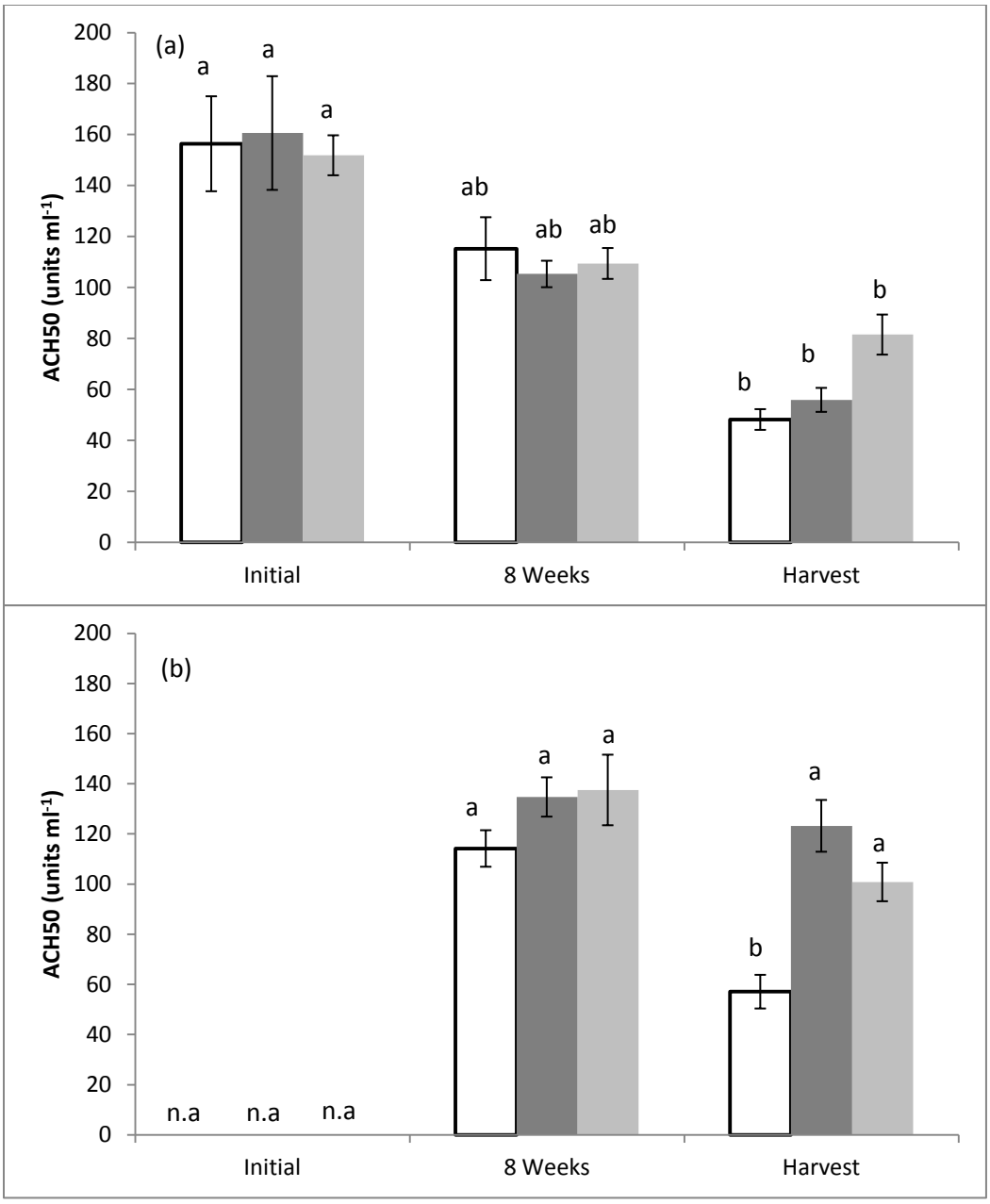

Figure 2.11 Alternative Complement activity (ACH50) from transfer to harvest shown as mean ± se for each treatment: Control (□), Vitamin (■) and Immunostimulant (■) for tows (a) 1 and (b) 2. (n=7 at initial and n=20 for 8 weeks and harvest). Different letters denote significant differences at p<0.05. n.a. denotes samples which were not available.

2.4 Discussion

Supplements of vitamins (predominantly C and E) or vitamins and immunostimulants (β-glucans and nucleotides) were delivered to ranched *T. maccoyii* over the first 12-weeks post transfer into grow-out pontoons. Elevated vitamin C content within tail muscle at 8-weeks post transfer and elevated vitamin E content at harvest provides evidence that vitamin supplementation was biologically available to *T. maccoyii*. This result is consistent with D'Antignana *et al.* (2008), who observed a 500 mg $fish^{-1}$ day^{-1} increase in dietary vitamin C and E over two months resulted in ~2 to 3x increase in ranched *T. maccoyii* muscle vitamin concentrations. The rapid decrease in muscle vitamin C concentration post supplementation has been observed by Thomas (2007) and again at harvest in the current study, and may be related to the water-soluble nature of vitamin C (Svirbely & Szent-Gyorgyi 1932) compared to the lipid soluble vitamin E (Weiss *et al.* 1962). Unfortunately, there was no test available to measure the biological availability of β-glucan and nucleotide supplementation however both were provided at biologically relevant concentrations.

Two main collective effects of vitamin C and E or vitamin and immunostimulant supplementation were found in this study: (1) reduction in plasma glucose at harvest and (2) increase in lysozyme activity at 8-weeks post transfer. Plasma glucose was collectively reduced at harvest for *T. maccoyii* in the Immunostimulant group. Plasma glucose can be elevated after feeding through the breakdown of carbohydrates (Halver and Hardy 2002) or from the stress-induced cortisol cascade, which releases the energy store of glycogen in liver and muscle (Halver & Hardy 2002). As there were no collective positive effects on other stress indicating variables (i.e. pH, osmolality, cortisol or lactate), it may suggest a supplement mediated digestive effect on *T. maccoyii*. Due to the limited sampling time points within this study and using relatively low stress capture methods such as baited hook-and-line and diver capture (ASBTIA, pers. comm.), this study may not have provided a good indicator of dietary supplementation impacts on stress response. Future work may need to use supplements in combination with a stress test to adequately test the stress related responses. Because no glucose effect was observed on *T. maccoyii* fed only vitamin supplement, this effect can be attributed to immunostimulants alone. In addition, increase in lysozyme activity at 8-weeks post transfer for both the vitamin C and E and the vitamin and

immunostimulant supplement fish was also observed. Lysozyme is a bactericidal enzyme, capable of breaking down gram-negative bacteria cell walls. An elevation in lysozyme activity can therefore be translated as an enhancement in immunocompentance. Several other studies have found an elevation in lysozyme activity following supplementation of vitamins in *S. salar* (see Hardie *et al.* 1990, Waagbø *et al.* 1993) and *Psetta maxima* L. (see Roberts *et al.* 1995). Due to the lack of differentiation in lysozyme activity between *T. maccoyii* fed additional vitamin C and E compared to *T. maccoyii* supplemented with vitamins and immunostimulants, the enhancement may be attributed to vitamins alone. There was no collective effect of the supplementation of the diet on performance (i.e. survival, condition index, and crude fat), parasite loads, hemaoglobin, stress (i.e. pH, osmolality, cortisol and lactate) or alternative complement activity.

Although there were few effects of treatments observed, which were common for both tows, several tow specific effects were found. Enhanced performance as measured through increased survival was observed in *T. maccoyii* from tow 1 fed additional vitamin C and E. Vitamin mediated enhanced survival has also been observed in *S. salar* (see Waagbo *et al.* 1993), *Mystus gulio* (Hamilton) (see Anbarasu & Chandran 2001), and *O. mykiss* (see Anggawati-Satyabudhy *et al.* 1989, Pearce *et al.* 2003). *Cardicola forsteri* prevalence was also reduced in *T. maccoyii* fed vitamin C and E supplemented diet in one of the tows. The number of *Caligus* spp. per *T. maccoyii* was also reduced. This effect was only observed in one tow. No reduction in *C. forsteri* was observed in *T. maccoyii* supplemented with vitamins and immunostimulants, suggesting a dampening effect of immunostimulants on the effects of vitamins to reduce *C. forsteri* loads. There was no such dampening effect on *Caligus* spp. load with the vitamin and immunostimulant supplemented *T. maccoyii* having equally lower abundance than vitamin only supplemented *T. maccoyii*. *Salmo salar* showed a 37.8% reduction in the mean number of sea lice, *Lepeophtheirus salmonis* per fish when given a diet supplemented in nucleotides and β -glucans (Burrells *et al.* 2001a), yet no such effect was observed in this study. The lack of clear trends between supplementation of vitamins and/or vitamins and immunostimulants significantly changing parasite loads monitored in this study may be due to the low prevalence and abundance of parasites compared to past seasons (Munday *et al.* 2003, Deveney *et al.* 2005, Aiken *et al.* 2006, Hayward *et al.* 2008), therefore a treatment effect could have been masked by both sampling schedule as well as the high natural variability

expected when using wild fish in commercial farming conditions. Due to the timing of the sampling in this experiment, epizootics may have occurred but were not observed. For example, in other *T. maccoyii* ranched within the TOFZ in 2009 *Caligus* spp. increased in numbers 2-3 weeks post transfer, and subsequently declined to zero 6 to 8-weeks post transfer. Therefore the loads of this parasite may have been missed due to the sampling schedule (Hayward *et al*. 2010). There was a reduction in stress as measured through plasma cortisol, for vitamin and immunostimulant supplemented *T. maccoyii* at 8-weeks, but only for one tow. Enhanced immune response, mediated through alternative complement activity (ACH50) was observed at harvest for both the vitamin and the vitamin and immunostimulant supplemented *T. maccoyii*. ACH50 aids the breakdown of pathogen cell walls, therefore increased activity can be directly related to enhanced immunocompetance. There was no additional effect of immunostimulant supplementation on ACH50 response; therefore it may be assumed ACH50 was vitamin enhanced. Vitamin C and E enhancement of complement activity has also been observed in *S. salar* (see Hardie *et al*. 1990; Waagbø *et al*. 1993), *Ictalurus punctatus* (Rafinesque) (Li & Lovell 1985, Li *et al.* 1993), gilthead seabream *Sparus aurata* L. (see Ortuno *et a.l* 1999, 2003, Montero *et al.* 1999), and *O. mykiss* (see Pearce *et al.* 2003). Although there were treatment effects on hemaoglobin, the concentration never exceeded the acceptable normal range of 13-21g/dL (Rough *et al.* 2005, Clark *et al.* 2008), therefore not considered physiologically significant.

The cause of tow specific treatment effects, may be attributed to many unique differences between the health and life history of the *T. maccoyii* in each tow, as well as conditions during each tow. Before one year post hatch, juvenile *T. maccoyii* first appear in Southwestern Australia, and begin to migrate south toward New Zealand through the Great Australian Bight from two to four years of age (Grewe *et al*. 1997). At this age, the immature fish form large surface schools (Cowling & Gunn 1999) and forage nearshore (Hearn & Polacheck 2003). As the *T. maccoyii* mature, schools reduce in number and *T. maccoyii* forage in more open water (Hearn & Polacheck 2003). Approximately 2 to 4 year old *T. maccoyii* are the perfect age for ranching, both in terms of ease of catch and economic value of return (Dell & Hobday 2008). Capture in the Australian *T. maccoyii* ranching industry consists of purse seining large schools of ~3 year old juvenile *T. maccoyii*, at times combining several schools to a desired tonnage, and towing these *T. maccoyii* on average over a 2-3 week period to Spencer Gulf, near Port Lincoln. At this point each school

essentially has its own discrete life history, genetic composition, and health. The natural mixing rate of *T. maccoyii* between schools is unknown, but mixing schools within a tow is fairly common. Tow conditions vary by company, weather conditions and in-water conditions, presence of baitfish and/or feeding frequency, and predation. Due to the large number of factors affecting towed *T. maccoyii*, once transferred to the grow-out pontoons, each tow of *T. maccoyii* may react differently to ranching. Performance, in terms of survival and condition index, can vary greatly between tows (ASBTIA, pers. comm.). Previous research on vitamin and immunostimulant supplementation has occurred on species of fishes which are cultured from eggs through grow-out with relatively uniform life histories, health, and husbandry. This is the first study to suggest these three parameters, life history, health, and husbandry, may affect the effectiveness of vitamin and immunostimulant supplementation.

The biological availability of immunostimulants β-glucans and nucleotides was not directly measured in this study, but can be inferred through effects on performance, stress, and immune response variables. A general reduction in plasma glucose can be attributed to immunostimulant supplementation alone. There was also a suggested dampening effect on the ability of vitamins to reduce *C. forsteri* loading in one tow of *T. maccoyii*. A decrease in plasma cortisol at 8-weeks post transfer in one tow of *T. maccoyii* was another tow-limited effect. The evidence for biological availability of immunostimulants is not overwhelming. It has been suggested nucleotide digestion may not occur in some fishes (Roald, 1978). It may be that only the inosine monophosphate (IMP) form, not inosine, is biologically available to fishes (Ikeda *et al*. 1991, Kubitza *et al*. 1997, Li & Gatlin 2006). Similarly, there are both soluble and particulate β-glucans commercially available, but only the soluble form has been shown to be absorbed by the intestines of fishes (Dalmo & Bogwald 2008). The stimulating effect of both β-glucans and nucleotides varies with the composition of the ingredient, route of administration, antigen source, environment, dose and timing (Ainsworth *et al*. 1994, Dalmo & Bogwald 2008) and age/size related response, especially in sub-adults where available research is insufficient. Therefore, the lack of immunostimulant effectiveness in this study may have been a case of insufficient dosing, timing, incorrect formulation, or the inability of *T. maccoyii* to incorporate exogenous β-glucans and nucleotides.

Several interesting correlations were found between variables within these results. Lysozyme activity was enhanced during supplementation of vitamins, when vitamin C within muscle was also elevated. With a decline in vitamin C reserves, there is also a decline in lysozyme activity at harvest. This may suggest lysozyme activity is limited by vitamin C availability. Direct causation between vitamin C and immune response cannot be determined in this study and will require further research. An interesting correlation was observed between tow specific, vitamin induced reduced prevalence of adults *C. forsteri* at harvest and decreased mortality. It is unknown if these two observations are linked in a causal relationship without further experimentation.

Important observations were also noted, unrelated to treatment effects. There was a trend for the highest prevalence of *C. forsteri* at the end of the trial and not at 8-weeks post transfer as in previous studies. These observations occurred in all treatments, therefore due to influences outside this experiment.

The observation of acidosis within one grow-out pontoon is assumed to be due to outside factors other than these experimental dietary treatments. Acidosis has not been previously reported due to vitamin supplementation, but has been observed in extreme cases of stress due to occurrences of suboptimal water quality (Parks *et al.* 2007), trauma (Packer & Sunkin 1979), capture (Brill *et al*. 2008), or extended activity (Wood *et al*. 1977).

This study demonstrated several enhancements in immune response, health, and performance which vitamin and immunostimulant supplementation can elicit on ranched *T. maccoyii*. The effects were not uniform throughout all ranched *T. maccoyii*, resulting in a low predictability of response. Further studies are needed to investigate the mechanism behind varied response, optimal dosing and timing of delivery, as well as composition of immunostimulants.

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CHAPTER 3:

EFFECTS OF THE FIRST TWO MONTHS OF RANCHING ON THE HEALTH OF SOUTHERN BLUEFIN TUNA *THUNNUS MACCOYII*

Kirchhoff, N.T., Leef, M.J., Ellis, D., Purser, J., Nowak, B.F., (2011). Effects of the first two months of ranching on the health of Southern Bluefin Tuna *Thunnus maccoyii*. Aquaculture 315: 207-212.

Abstract

A weekly monitoring project was designed to improve our understanding of the dynamics of southern bluefin tuna health, immune response, and performance during the first two months of ranching. In addition, results were compared to the data for wild fish to highlight any effects of captivity. Weight, length, condition index, hemaoglobin concentration, and immune response were all found to change significantly over this period. SBT were found to be relatively healthy prior to a mortality event which resulted in a cumulative mortality of 8.5%. The mortality event was associated with decreased hemaoglobin concentrations and changes in immune response.

3.1 Introduction

Ranching, or the process of temporarily holding wild fish in sea cages to fatten them on baitfish, was developed by the Southern Bluefin Tuna (SBT) industry of South Australia in 1991 in response to a severe reduction in total allowable catch. Ranching enhances the fat content and size of captured SBT, allowing the industry to obtain premium market value for their catch, thereby maintaining profitability with decreased total catch. This technology has since been adopted by other bluefin tuna industries worldwide (CCSBT 2010). In Australia, schools of 2-4 year old wild SBT are captured by purse seine and carefully towed back to the Tuna Offshore Farming Zone (TOFZ) in Spencer Gulf near Port Lincoln, South Australia where they are transferred into several grow-out pontoons. Several tows of SBT are captured from December to April each year, with each distinct tow of fish collectively called a cohort. For three to six months, SBT are fattened on baitfish and/or manufactured feed until they are harvested for the high end sashimi market (ASBTIA, pers. comm.).

Ensuring the health and wellbeing of ranched SBT is not only important for optimum production, but for the conservation and sustainability of the fishery. Over the past few decades, a period of increased mortality has been identified to occur during the first two months of ranching, resulting in an average cumulative mortality of 2-12% (ASBTIA, pers. comm.). A large amount of variation both within and between cohorts of fish with regard to their health, immune response, and performance has been reported (Watts *et al.* 2002, Aiken *et al.* 2008, Kirchhoff *et al.* 2011), making investigations into the cause of this increased mortality difficult. One possible hypothesis is that considerable microscale changes may occur thereby magnifying variation when sampling is pooled over large time periods or between cohorts.

The present study aimed to describe the microscale, i.e. weekly variation in health of SBT for the first two months of ranching. Ranched SBT were sampled between week four and nine of ranching, thereby allowing fish to acclimate to captivity while also encompassing the entire mortality event. In addition, differences between ranched and wild fish at the earliest stages of ranching will also be described. Health was assessed through the monitoring of survival (mortality), parasite loads, hematology, and humoral immune variables. While this study was limited to opportunistic sampling during commercial

harvest, the results increased our understanding of variation in health, immune response, and performance parameters within a cohort of ranched SBT.

3.2 Materials and Methods

3.2.1 Experimental Fish and Study Design

Approximately 11,900 wild SBT were captured by purse seine in the Great Australian Bight from 3-6 December 2009. Following transport to the TOFZ in a towing pontoon, SBT were transferred into three grow-out pontoons on 26 December 2009. SBT were stocked at an initial density of 2.376kg m^{-3}. SBT were fed sardines at an average rate of 1.28kg SBT^{-1} day^{-1} for their entire ranching period.

In addition, 22 wild SBT were sampled in the Great Australian Bight prior to purse seining, from 10 to 21 January 2010. These wild fish were used as a baseline of 'normal' health status, yet it is significant to mention these wild fish were not taken from the same school nor at the same time the ranched fish were captured.

3.2.2 Sample Collection

3.2.2.1 Field Collection

Samples were opportunistically collected from week 4 to week 9 post-transfer during commercial harvest; therefore sample size varied with commercial demand. All fish were captured using a baited hook and line. Once landed on the boat, SBT were immediately spiked in the head, brain removed using a 'Taniguichi tool' (core) and a wire placed down the spine to destroy the upper spinal nerves. Total time between capture and killing of each SBT was less than one minute. Length and weight were recorded for all SBT at the time of sampling, after the gills and viscera were removed. Condition index was calculated for each SBT using the formula: [gilled and gutted weight (kg) /0.87] / [length (m) ^3] (Hayward *et al.* 2010), which converts SBT weight to whole weight. External metazoan parasites were then quantified from both the skin and gill arches by the naked eye. Parasites were counted on gill arched after the gills were removed from the fish and dissected. As all the significant metazoan gill parasites of SBT are large

size, counts using naked eye are considered to be accurate and acceptable for a routine use (Deveney et *al.* 2005, Hayward *et al.* 2007). All lice visible to the naked eye were collected; any additional lice remaining on tuna surfaces were then detected using a technique described by Hayward *et al.* (2010). Immediately after external surface examination, whole blood was collected from the severed pectoral artery behind the pectoral recess in 2-9ml Vacutainer ® tubes (BD, USA), one heparinized and one non-heparinized, and placed on ice. Blood was collected within 3 minutes of fish capture. The heart was placed in a waterproof tub, the visceral organs were placed in a waterproof bag and both stored on ice. The SBT carcass was then placed in ice slurry.

The wild SBT were captured, killed, and processed identical to the ranched SBT. Weight was not recorded for wild SBT due to unstable sea conditions at the time of sampling.

3.2.2.2 Laboratory Processing

The heparinized vial of whole blood was used for whole blood and plasma aliquots. Three 500µl aliquots of whole blood were transferred into 1.5ml plastic tubes and frozen at -20°C. The remaining blood was centrifuged at 3000xg at 4°C for 5min. Blood plasma was aliquoted into five 1.5ml plastic tubes, and frozen at -20°C. No plasma was collected from wild SBT. The non-heparinized vial of whole blood was used for serum collection. Vials were stored upright at 4°C for 24h, centrifuged at 1000xg at 4°C for 5min, and serum aliquoted into three-1.5ml tubes. Serum samples were stored at -20°C. Hearts of ranched SBT were dissected 2–4h after removal from the carcass and flushed with physiological saline to dislodge any adult *Cardicola forsteri* (see Aiken *et al*. 2006). Flushes were then poured into Petri dishes and examined for the presence of adults using a dissecting microscope. Wild SBT hearts were stored frozen at -20°C. Before processing they were thawed and then flushed with physiological saline and flushes examined as described above.

3.2.3 Blood Variables

3.2.3.1 Hematology

Hemaoglobin concentrations were determined from whole blood aliquots using the cyanometahemaoglobin assay based on Brown (1984) modified by Kirchhoff *et al*. (2011).

Whole blood plasma glucose and lactate were measured using Accu-Chek ® Advantage II and Accutrend ® Plus by Cobas, respectively. The pH of blood plasma samples was measured using a Minilab Isfet pH meter Model IQ125 (IQ Scientific, USA). Blood plasma osmolality was determined using a Vapro© Model 5520 vapour pressure osmometer (Wescor Inc., Logan, Utah, USA). Blood pH and osmolality was determined using serum for the wild samples, as they were determined to have equivalent values from both serum and plasma.

3.2.3.2 Humoral Immune Response

Blood serum was analyzed in triplicate for lysozyme activity and alternative complement activity. Lysozyme activity was measured using a method based on that described by Carrington and Secombes (2007) modified by Kirchhoff *et al*. (2011). Blood serum alternative complement activity was measured using a modified Yano (1992) method as described by Kirchhoff *et al*. (2011).

3.2.4 Statistical analyses

Parasite infections were characterized by prevalence (the number of host infections as a proportion of the population at risk) and mean abundance (the average number of parasites in all hosts) (Bush *et al*. 1997). Sterne's exact 95% confidence intervals were calculated for prevalence, and 95% bootstrap confidence intervals (with 2000 replications) were calculated for mean abundance, using the software 'Quantitative Parasitology 3.0', supplied by Reiczigel & Rózsa (2005). The prevalence and mean abundance for each species for each pontoon were compared with other treatments and ranching durations in a pair-wise fashion. Given the high total number of pairwise comparisons, $\alpha = 0.01$ was regarded as significant for these statistics.

All blood and physical condition results were interpreted using the R 2.8.1 statistical package (© 2008, The R Foundation for Statistical Computing). Effect of ranching duration, in weeks (post transfer), was determined for each immune variable using an ANOVA. Wild fish were included in the analysis for comparison as a pre-ranching control. Data expressed for week 6 of ranching were included for analysis but the results must be taken with caution due to small sample size. The assumption of homogeneity of variances was checked by the residual plot and Bartlett test. Plasma pH was log 10 transformed for all statistical analyses. The Tukey HSD post-hoc test was applied at a significance level of α=0.05, to determine differences between the explanatory variables.

Spearman rank correlation coefficients were calculated using R 2.8.1 statistical package (© 2008, The R Foundation for Statistical Computing) for the relationship between Hb, lysozyme, and alternative complement activity with *C. forsteri* parasite counts, for all fish examined. A significance level of α=0.01 was applied.

3.3 Results

3.3.1 Survival and SBT size characteristics

Absolute mortality, averaged for all three ranching pontoons, remained relatively low until week five post transfer (Figure 3.1). Mortality was elevated from week five to week seven with cumulative mortality between transfer and week twelve of ranching equalling 8.5%.

The weight of SBT samples increased ($F=4.2679$, $df=5,40$, $p=0.003$) between week four and week nine. SBT at week four and week seven were on average 7 kilograms lighter than SBT at week nine. There was no difference in the average weight between week nine and week five, six, or eight of ranching (Table 3.1). The length of SBT increased by approximately 10cm between week four and week nine of ranching ($F=3.5078$, $df=5,40$, $p=0.01$) (Table 3.1). The condition index (CI) of ranched fish was found to change within the first two months ($F=10.799$, $df=5,40$, $p<0.001$). CI decreased between week four and week five, remaining low until week nine when CI returned to a level not different from week four (Table 3.1).

Table 3.1 Mean ± SE for selected physical and blood parameters in southern bluefin tuna (*Thunnus maccoyii*) from the wild and week four to week nine of ranching: gilled and gutted weight (kg), fork length (cm), condition index (CI), plasma pH, plasma osmolality (mmol kg-1), and plasma lactate (mmol l-1). Different letters denotes statistical difference within a parameter over ranching duration at α=0.05. n.a. denotes samples which were not available.

	Wild (n=22)	Week 4 (n=8)	Week 5 (n=6/4blood)	Week 6 (n=2)	Week 7 (n=10)	Week 8 (n=10)	Week 9 (n=10)
Weight (kg)	n.a.	19.96±0.99^{b}	22.08±1.47^{ab}	26.50±2.50^{ab}	18.25±0.97^{b}	21.55±2.00^{ab}	26.79±1.92^{a}
Length (cm)	105.8±2.3^{ab}	99.8±1.9^{b}	108.8±2.7^{ab}	114.5±5.5^{ab}	101.3±1.2^{ab}	108.0±3.2^{ab}	109.7±2.5^{a}
CI	n.a.	21.86±0.52^{a}	19.59±0.43^{b}	20.30±1.01^{ab}	20.01±0.44^{b}	19.12±0.40^{b}	23.00±0.48^{a}
pH	7.78±0.03^{b}	7.68±0.07^{b}	7.75±0.10^{ab}	7.90±0.30^{ab}	7.99±0.03^{a}	8.01±0.05^{a}	8.02±0.06^{a}
Osmolality	439.7±17.1	390.2±3.8	379.0±9.4	417.0±21.0	417.5±15.0	397.0±1.6	450.4±6.1
Lactate	6.85±0.22	7.00±0.61	7.20±0.35	7.95±1.35	7.84±0.37	6.99±0.26	7.37±0.33

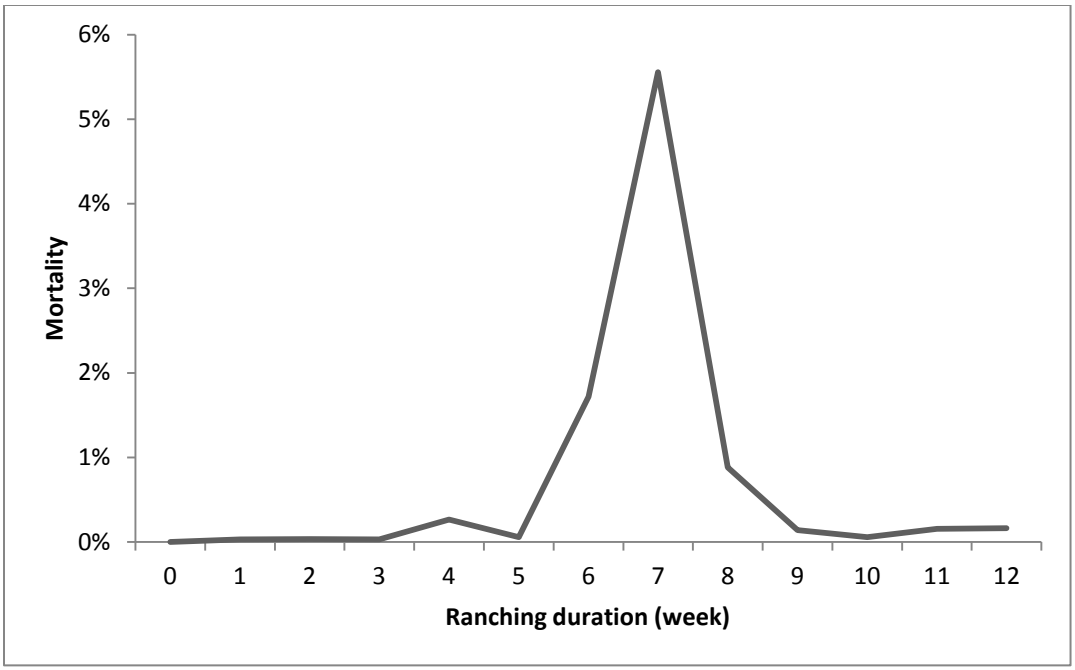

Figure 3.1 Weekly mortality of SBT from transfer into sea pontoons until week 12 of ranching.

3.3.2 Parasitology

Prevalence of *Caligus* spp. increased from 0% in wild SBT to a peak of 60% after five weeks of ranching, with no significant differences observed between other sampling times (Table 3.2). No difference was found in the mean intensity and abundance of *Caligus* spp. Prevalence of *Cardicola forsteri* increased from 0% in the wild to a peak of 100% after five weeks of ranching, with ranched fish having higher prevalence than wild at all sample times except for week 6 where sample size was low (Table 3.2). The mean abundance of *Cardicola forsteri* was 3.5 times higher at week 7 compared to week 9 of ranching ($t=3.239$, $p=0.009$), however no significant differences were observed between fish sampled at other times (Table 3.2). There was no statistically significant difference in mean intensity of *C. forsteri* between wild and ranched SBT. No difference was found in the prevalence, mean abundance and intensity of gill parasites (*Hexostoma thynni, Pseudocycnus appendiculatus,* or *Euryphorus brachypterus*) of SBT (Table 3.2).

Table 3.2 Summary for prevalence (95% confidence interval) and mean abundance (95% confidence interval) and mean intensity (95% confidence interval) for five common parasites of *Thunnus maccoyii* from from the wild and week four to week nine of ranching. 'na' is denoted when confidence interval could not be calculated. Different letters denotes statistical difference within a parasite over ranching duration at α=0.01.

Ranching Duration (weeks)	Prevalence (%)	Mean Abundance	Mean Intensity
Caligus spp.			
Wild (n=22)	0.0 (15.2-0.0)[b]	0.00 (0.00-0.00)	0.00 (na)
4 (n=8)	25.0 (56.2-6.9)[ab]	0.38 (1.00-0.00)	1.50 (1.50-1.00)
5 (n=6)	60.0 (92.3-18.9)[a]	0.60 (0.80-0.00)	1.00 (1.00-1.00)
6 (n=2)	0.0 (77.6-0.0)[ab]	0.00 (0.00-0.00)	0.00 (na)
7 (n=10)	0.0 (29.1-0.0)[ab]	0.00 (0.00-0.00)	0.00 (na)
8 (n=10)	10.0 (44.6-0.5)[ab]	0.10 (0.30-0.00)	1.00 (na)
9 (n=10)	0.0 (29.1-0.0)[ab]	0.00 (0.00-0.00)	0.00 (na)
Cardicola forsteri			
Wild (n=22)	0.0 (15.2-0.0)[b]	0.00 (0.00-0.00)[b]	0.00 (na)
4 (n=8)	87.5 (99.4-50.0)[a]	5.00 (8.38-2.63)[ab]	5.71 (9.43-3.43)
5 (n=6)	100.0 (100-58.9)[a]	3.50 (5.33-1.67)[ab]	3.50 (5.33-1.67)
6 (n=2)	50.0 (97.5-2.5)[ab]	2.00 (na)	4.00 (4.00-0.00)
7 (n=10)	90.0 (99.5-55.5)[a]	2.10 (2.70-1.30)[a]	2.33 (2.89-1.67)
8 (n=10)	70.0 (91.3-38.1)[a]	1.80 (2.80-0.80)[ab]	2.57 (3.57-1.57)
9 (n=10)	40.0 (70.9-15.0)[a]	0.60 (1.00-0.10)[b]	1.50 (1.75-1.00)
Hexostoma thynni			
Wild (n=22)	27.3 (50.0-12.6)	1.05 (2.82-0.27)	3.83 (7.33-1.17)
4 (n=8)	25.0 (63.5-4.6)	1.00 (3.00-0.00)	4.00 (4.00-2.00)
5 (n=6)	16.7 (58.9-0.9)	0.17 (0.50-0.00)	1.00 (na)
6 (n=2)	50.0 (97.5-2.5)	2.50 (5.00-0.00)	5.00 (na)
7 (n=10)	30.0 (61.9-8.7)	1.60 (3.80-0.20)	5.33 (7.33-2.00)
8 (n=10)	30.0 (61.9-8.7)	0.80 (2.30-0.10)	2.67 (4.00-1.00)
9 (n=10)	0.0 (29.1-0.0)	0.00 (na)	0.00 (na)
Pseudocycnus appendiculatus			
Wild (n=22)	27.3 (50.012.3)	0.36 (0.64-0.14)	1.33 (1.66-1.00)
4 (n=8)	12.5 (50.0-0.6)	0.13 (0.25-0.00)	1.00 (na)
5 (n=6)	0.0 (41.1-0.0)	0.00 (0.00-0.00)	0.00 (na)
6 (n=2)	0.0 (77.6-0.0)	0.00 (0.00-0.00)	0.00 (na)
7 (n=10)	30.0 (61.9-8.7)	1.30 (4.60-0.20)	4.33 (7.33-1.00)
8 (n=10)	20.0 (55.4-3.7)	0.30 (0.80-0.00)	1.50 (1.50-1.00)
9 (n=10)	20.0 (55.4-3.7)	0.70 (1.60-0.00)	3.50 (3.50-3.00)
Euryphorus brachypterus			
Wild (n=22)	59.1 (77.8-38.3)	6.14 (14.36-2.45)	10.38 (20.77-4.15)
4 (n=8)	37.5 (71.1-11.1)	1.13 (2.50-0.13)	3.00 (3.00-1.00)
5 (n=6)	50.0 (84.7-15.3)	1.17 (2.17-0.17)	2.33 (2.99-1.00)
6 (n=2)	0.0 (77.6-0.0)	0.00 (0.00-0.00)	0.00 (na)
7 (n=10)	30.0 (61.9-8.7)	0.50 (1.00-0.00)	1.67 (2.00-1.00)
8 (n=10)	40.0 (70.9-15.0)	1.30 (2.80-0.30)	3.25 (5.00-1.25)
9 (n=10)	10.0 (44.6-0.5)	0.10 (0.30-0.00)	1.00 (na)

3.3.3 Blood

Hb concentration rapidly declined and subsequently recovered between wild and week nine of ranching ($F=19.769$, $df=6,59$, $p<0.001$) (Figure 3.2). Hb declined to a minimum of 14.3 ± 1.09 at week 7 of ranching, lower than week four, eight and nine by approximately 4g dl^{-1}. Hb concentration was 25% higher in wild SBT when compared to pooled data from SBT sampled from week four to week nine of ranching. While overall Hb concentration remained within acceptable normal range of 13-21 g dl^{-1} (Rough *et al*, 2005; Clark *et al*, 2008), the rapid drop over only a few weeks may suggest the occurrence of a significant physiological disturbance.

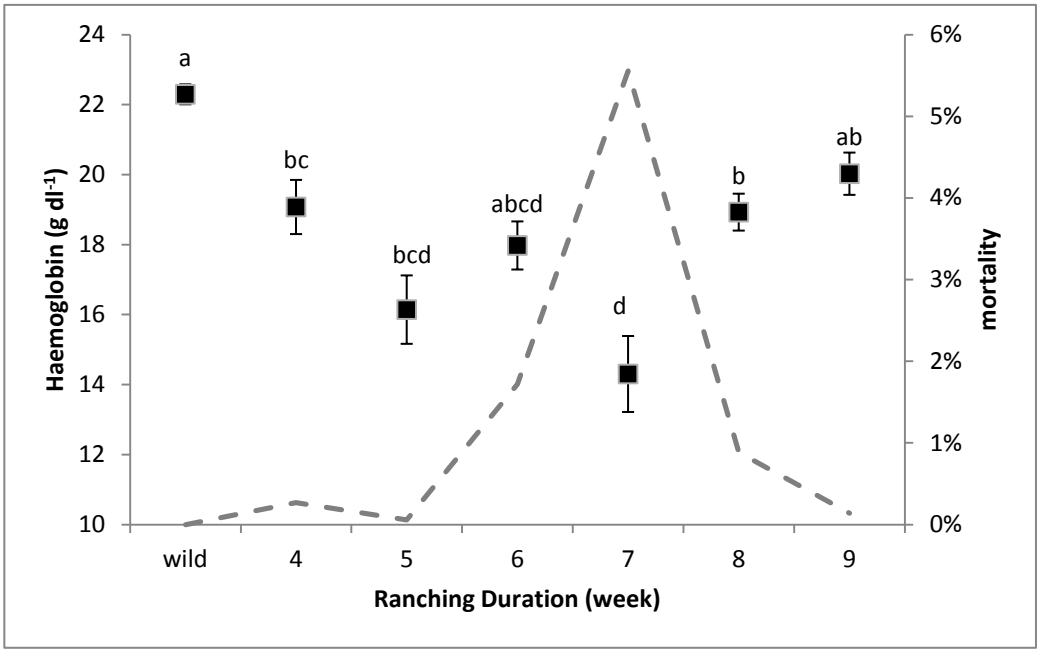

Figure 3.2 Hemaoglobin concentration ± SE in whole blood collected from wild and ranched SBT from week four to week nine of ranching. Different letters denote statistical differences at α=0.05. Concurrent weekly mortality (%) is denoted by a dotted line.

Blood glucose was 30% higher at week 7 of ranching compared to the blood glucose of wild SBT ($F=2.9366$, $df=6, 59$, $p=0.0142$) (Figure 3.3). There was no difference at any other time. When ranching data were pooled, ranched fish had 30% higher glucose than wild fish ($t=3.46$, $df=64$, $p<0.001$).

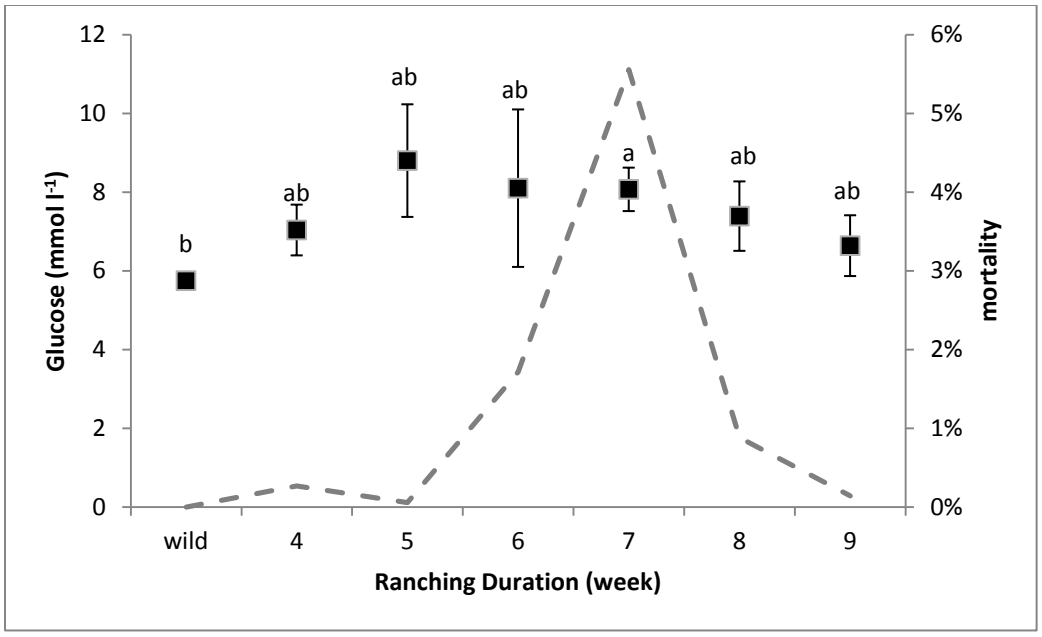

Figure 3.3 Blood glucose ± SE in whole blood collected from wild and ranched SBT from week four to week nine of ranching. Different letters denote statistical differences at α=0.05. Concurrent weekly mortality (%) is denoted by a dotted line.

Plasma pH was elevated from week 7 to 9 of ranching compared to the blood plasma pH of wild SBT and week 4 ($F=6.727$, $df=6,59$, $p<0.001$) (Table 3.1). Plasma pH remained within a very narrow range, therefore no physiological significant change in pH was found. No difference was found for blood osmolality ($F=2.243$, $df=6,59$, $p=0.051$) or lactate concentration ($F= 1.1362$, $df=6,59$, $p=0.3529$, Table 3.1).

3.3.4 Humoral Response

Lysozyme concentration in blood serum of ranched SBT was significantly elevated compared to the wild SBT at all ranching times ($F=9.9248$, $df=6,59$, $p<0.001$) (Figure 3.4). Mean lysozyme concentration was 8 times greater than that of wild SBT at all weeks except week five where lysozyme concentration was found to be as high as 15 times that of wild SBT.

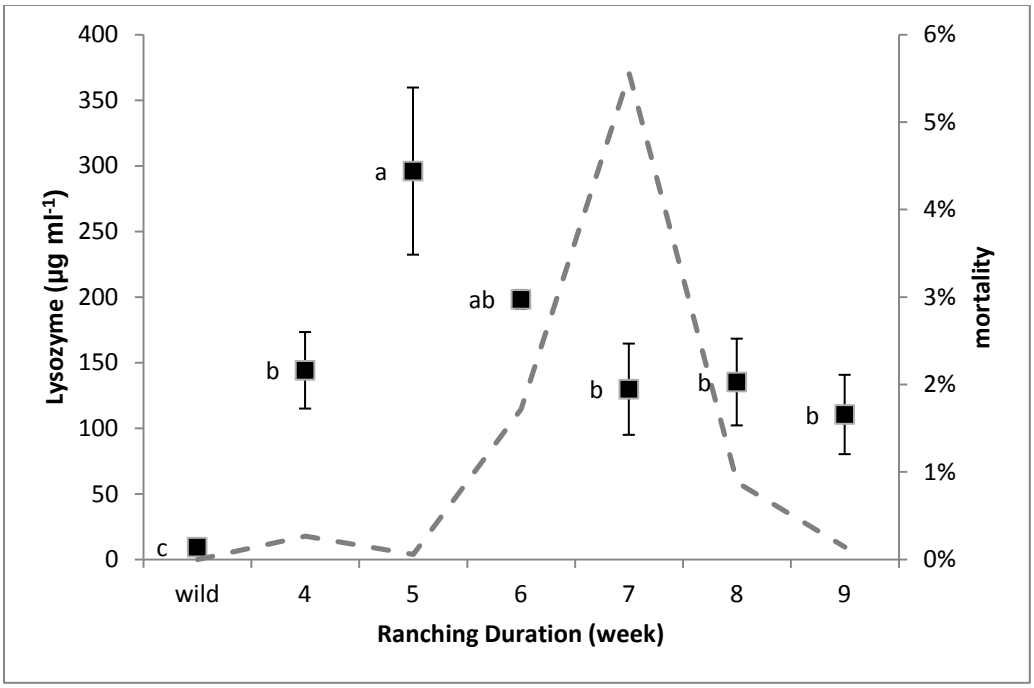

Figure 3.4 Blood lysozyme ± SE in serum collected from wild and ranched SBT from week four to week nine of ranching. Different letters denote statistical differences at α=0.05. Concurrent weekly mortality (%) is denoted by a dotted line.

Alternative Complement activity (ACH50) was 2 times higher for SBT sampled at week 5 of ranching compared to wild fish and those at week 8 and 9 of ranching (F=4.23, df=6,59, p=0.0014) (Figure 3.5).

3.3.5 *C. forsteri* correlations

The intensity of *C. forsteri* infection was not correlated with Hb concentration (S=15648.30, p=0.5068), lysozyme activity (S=9584.697, p=0.2075), nor alternative complement activity (S=9584.697, p=0.032).

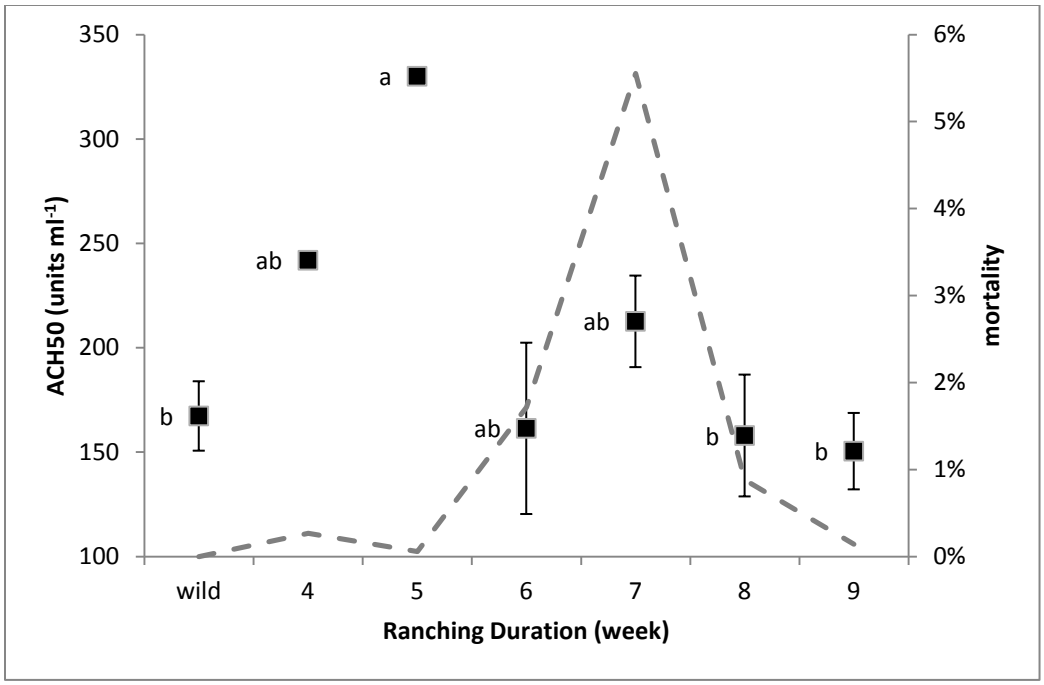

Figure 3.5 Blood alternative complement activity (ACH50) ± SE in serum collected from wild and ranched SBT from week four to week of ranching. Different letters denote statistical differences at α=0.05. Concurrent weekly mortality (%) is denoted by a dotted line.

3.4. Discussion

There were no significant changes in health and condition of ranched SBT on a weekly timescale in the first two months of ranching. Weight, length, condition index, hemaoglobin concentration, lysozyme activity, and alternative complement activity were all found to change over this period. In addition, differences between wild and ranched SBT occurred as early as the fish were sampled for the first time - week four of ranching, earlier than previously believed.

Since sampling spanned the entire mortality event, conclusions on changes in SBT health can be put into context. An elevation in lysozyme and alternative complement activity just prior to the initiation of mortality suggests ranched SBT were not immunocompromised and could produce an innate immune response. In addition, the lack of difference between wild and ranched SBT in plasma pH, osmolality, and lactate also support the conclusion that ranched SBT were relatively healthy prior to the event. The results also suggest that the cause of the mortality event may be acute rather than chronic. The peak in

mortality was correlated with reduced Hb concentration and the peak in abundance of *C.forsteri*. There was also a significant drop in lysozyme and alternative complement activity associated with the duration of the mortality event, suggesting utilization by the agent which caused the mortality and/or a decrease in production.

The cause of the elevated humoral response and its rapid depletion is unknown, but links to the mortality event strongly suggests a correlation. Elevated lysozyme was found in association with increased *C. forsteri* infection intensity (Hayward *et al.* 2010). However, no correlation was found between lysozyme or alternative complement activity and *C. forsteri* infection intensity in this study. This may be due to much lower infection intensities than those reported by Hayward *et al.* (2010). Rapid lysozyme depletion was found in association with *Enteromyxum leei* infection of gilthead seabream, *Sparus aurata,* (see Sitjá-Bobadilla *et al*. 2008), where lysozyme activity dropped from 280units/ml to near 0 over 113 days of infection. Complement activity has also been associated with blood fluke infections. Total complement activity was found to change in *Sanguinicola inermis* infection of carp, *Cyprinus carpo*, where total complement activity increased 2.7x at week five post infection compared to week four and control levels. Total complement activity was found to return to normal at week seven post-infection (Roberts *et al.* 2005). The changes in alternative complement and lysozyme activity found in this study are consistent with those found in Roberts *et al.* (2005). Roberts *et al.* (2005) suggested changes in complement activity may be related to an increase in the number of macrophages in the spleen and pronephros which occurs at the same time in infection, as well as perhaps a consumption of complement by *S. inermis* adults. The peak in complement activity was correlated with commencing of egg production, which occurs at approximately 30 days post-infection in *S. inermis* infections (Roberts *et al.* 2005). It may be that both alternative complement and lysozyme activity are responding to the commencing of egg production of *C. forsteri* which is estimated to occur at 30 days post-infection (Aiken *et al*. 2009). Santoro *et al. (*1980) found a direct correlation between elevated complement levels and *S. mansoni* egg infection intensity in humans. Descriptions of the humoral immune response to the egg stage of *C. forsteri* infection are currently underway.

An association between peak *C. forsteri* abundance and peak mortality is consistent with previous reports (Hayward *et al.* 2010). Although few digenean species have been found to cause mortalities to their host, there are some rare cases reported within the literature: in 1966 and 1984, a *Bucephalus polymorphus* infection led to the mortality of cyprinids in France, Poland, and Germany (Hoffmann *et al.* 1990); in 1979 through 1982, a *Uvulifer ambloplitis* infection led to the mortality of bluegill sunfish, *Lepomis macrochirus*, in the USA (Lemley & Esch 1984); in 1993, a *Paradeontacylix grandispinus* and/or *P. kampachi* infection led to the mortality of 50-80% of the captive *S. dumerili* in Japan (Ogawa & Fukudome 1994); from 1997 to 1998, a *Centrocestus formosanus* infection led to the mortality of fountain darts, *Etheostoma fonticola*, in the USA (Mitchell *et al.* 2000); and finally, in 2000 two unidentified bucephalids killed two unidentified cyprid species in Japan (Bullard & Overstreet 2008). All these rare cases of digenea induced mortality occurred in combination with either environmental stress or a period of intense infection; therefore digenean infection alone was not the cause. In addition, all cases were due to the presence of the larval or egg stage of infection, rather than adult presence. Presently, there is no conclusive evidence of the link between *Cardicola forsteri* infection and mortality.

Unlike Hayward *et al.* (2010), no correlation was found between *C. forsteri* intensities and Hb concentrations. This may also be due to the much lower infection intensities. Maximum infection intensity was 14, compared to 441 flukes per SBT found by Hayward *et al.* (2010). The lack of correlation may also suggest that the causal agent for decline in Hb may be something other than *C. forsteri* infection, and correlations found in the previous study may have been spurious due to the drop in Hb and peak of *C. forsteri* infection occurring synchronously but caused by different factors.

The observations of elevated *Caligus* sp. infection at week five of ranching and its rapid decline is consistent with Hayward *et al* (2008) and does not seem to be associated with mortality.

The goal of a fattening operation is to increase fish weight, therefore condition and market value. Yet an indirect consequence of a continuously rich diet may be prolonged hyperglycemia. When non-insulin dependent mice were fed a high-fat diet, they developed hyperglycemia and obesity (Ikemoto *et al*. 1996). Fish are naturally predisposed to persistent hyperglycemia, mimicking non-insulin dependent diabetes

(Wilson 1994). Further investigations need to be completed on ranched SBT to diagnose clinical hyperglycemia and determine if it impacts their health.

It can be concluded that SBT acclimate quickly to ranching, i.e. within a month post transfer, and are moderately healthy prior to an acute mortality event. This study illuminated several new signs associated with the annual mortality event and highlighted new areas of investigation into its cause. Further research into the cause of the annual mortality event should investigate the cause for a drop in Hb, the cause of rapid changes in immune response, and the effects of the egg stage of *C. forsteri* infection. In addition, future studies comparing cohorts of fish should correct for the variation related to each week of ranching.

3.5 Acknowledgements

We thank the following people for their investment of resources and time in the completion of this study: Victoria Valdenegro, Karine Cadoret, Mathew Evans and Daniel Pountney of the University of Tasmania Aquatic Animal Health Group; Kirsty Rough, and Claire Webber of ASBTIA; the staff of the Lincoln Marine Science Centre; and Anthony Ellin, Robbie Staunton, and the vessel skippers and crews of Stehr Group. This project was funded by the Fisheries Research and Development Corporation of Australia.

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CHAPTER 4:

MOVING CAGES FURTHER OFFSHORE: EFFECTS ON SOUTHERN BLUEFIN TUNA, *T. MACCOYII*, PARASITES, HEALTH AND PERFORMANCE

Kirchhoff, N.T., Rough, K.M., Nowak, B.F., (2011). Moving cages further offshore: effects on southern bluefin tuna, *T. maccoyii*, parasites, health, and performance. PLoS ONE 6: e23705.

Abstract

The effects of offshore aquaculture on SBT health (particularly parasitic infections and haematology) and performance were the main aim of this study. Two cohorts of ranched Southern Bluefin tuna (SBT) (*Thunnus maccoyii*) were monitored throughout the commercial season, one maintained in the traditional near shore tuna farming zone and one maintained further offshore. SBT maintained offshore had reduced mortality, increased condition index at week 6 post transfer, reduced blood fluke and sealice loads, and haematological variables such as hemaoglobin or lysozyme equal to or exceeding near shore maintained fish. The offshore cohort had no *Cardicola forsteri* and a 5% prevalence of *Caligus* spp., compared to a prevalence of 85% for *Cardicola forsteri* and 55% prevalence for *Caligus* spp. near shore at 6 weeks post transfer. This study is the first of its kind to examine the effects of commercial offshore sites on farmed fish parasites, health and performance.

4.1 Introduction

Offshore aquaculture is in its infancy worldwide, yet commercial development is underway in numerous countries including USA, Ireland, Norway, Spain, Italy, Malta, Belgium, Scotland, UK, Japan, and Australia (OATP 2007). There are numerous factors which distinguish between a near shore and an offshore site, including location or hydrography, the in-water and above-water environment, the ease of access and associated operation logistics, yet no formal international definition has been made. In the context of this study, offshore was defined by reduced access (i.e. remoteness) and increased exposure to the environment, both in- and above-water. The attractiveness and potential benefits of moving aquaculture cages further from the shore are many, including fewer limits to the scale of operation, enhanced water quality, lower costs of environmental monitoring, reduced interaction with urban populations and inshore environmental concerns, and reduced disease risk (ERA 2009). In addition, moving cages from near shore to offshore sites may be necessary in the future due to the many anticipated effects of climate change (Hobday *et al.* 2008). Yet, many of these assertions have been insufficiently tested and the commercial feasibility of offshore development is presently unknown.

Offshore aquaculture has not been extensively developed for many reasons. Moving farther offshore is capital intensive, leading to increases in operation and servicing costs which need to be outweighed by potential performance benefits of the cultured species. There are also investment uncertainties related to the optimal configuration of sites, species most suitable to the exposed conditions, and lack of necessary technology (OATP 2007, ERA 2009). Technology does not just refer to strong farming structures cages; it also concerns advanced feeding techniques, communication, mortality retrieval systems, and monitoring systems, which allow management of stocks that are not easily accessible (OATP 2007). In addition, sufficient testing of the feasibility of offshore aquaculture also requires large baseline datasets in full commercial scale, which are absent in the current published literature.

Southern Bluefin Tuna (SBT) have been ranched in near shore cages in Port Lincoln, South Australia since 1991. In Australia, schools of 2-4 year old wild SBT are captured by purse seine and, carefully towed back to the Tuna Offshore Farming Zone (TOFZ) in Spencer Gulf near Port Lincoln, South

Australia where they are transferred into several grow-out cages and fattened on baitfish for three to six months. As a member nation of the Commission for the Conservation of the Southern Bluefin Tuna (CCSBT) and with sustainability in mind, the Australian SBT industry strictly adheres to catch quotas, quantified upon the arrival of a tow cage within the TOFZ, prior to the start of ranching. Large commercial scale baseline datasets have been collected for several decades concerning environmental monitoring, stock performance and health, and economic viability of ranched SBT maintained in the TOFZ (Australian Southern Bluefin Tuna Industry Association (ASBTIA) pers. comm.), enabling future research into alternative husbandry practices, such as site. In addition, current private investments made by the Australian SBT ranching industry into technology and operations infrastructure can be easily translated to the offshore environment. The aim of this project was to examine the feasibility of offshore versus near shore aquaculture using the ranching of SBT in Port Lincoln, South Australia as a case study. In this study, feasibility was measured through SBT health, i.e. parasite loads and haematology, and performance, i.e condition index and survival. Although cost cannot be directly considered in this study due to commercial confidentiality restrictions, economic implications are discussed.

4.2 Materials and Methods

4.2.1 Experimental Fish and Site Characteristics

Two different cohorts of SBT were captured by purse seine in the Great Australian Bight in February 2010. Each cohort was transported to the (TOFZ) in a separate towing cage. The near shore cohort of 9165 SBT was transferred on 14/3/2010 into three grow out cages and the offshore cohort of 7300 SBT was transferred on 15/3/2010 into three grow out cages. The near shore site was located at 34° 40.299' S, 136°04.708' E and the offshore site was located at 34° 44.409' S, 136°22.703' E (Figure 4.1). A complete description of the hydrology other environmental parameters for each site can be found in Table 4.1. Transfer procedures were identical between both sites, carefully stipulated and monitored by the Australian Fisheries Service as part of the CCSBT quota allocation guidelines. SBT were stocked at an initial cage density of 3.32kg m^{-3} for the near shore cohort and 3.37 kg m^{-3} for the off shore cohort.

Table 4.1 Comparison chart of the remoteness, above water environment and hydrology of the two farming sites, near shore in the Tuna Farming Zone (TFZ) and offshore. Distance from shore is represented as distance from port. Wind Speeds were measured using weather stations at Boston Island for the near shore site and an average of stations at Spilsby Island and Thistle Island (Figure 4.1).

Description Parameter		Near shore (TFZ)	Offshore	Reference
Remoteness	Distance from shore	16nm	25nm	
	Average number days per week site could be accessed by vessel	6-7 days	4-6 days	
Above water environment	Average wind speed	8.96knots	11.22knots	4
	Maximum wind speed	32.53knots	36.85knots	4
Hydrology	Water depth	20m	40m	
	Maximum significant wave height	0.8m	1.5-2m	5
	Tidal flow at 3.2m above seafloor	≤0.1m s-1	>0.5m s-1	6-7
	Flushing rate	2 days	<6hours	6
	Number of days per year sediments in top 10m of water column	5-10	<1	5
	Sediment environment	Depositional	Erosional	5

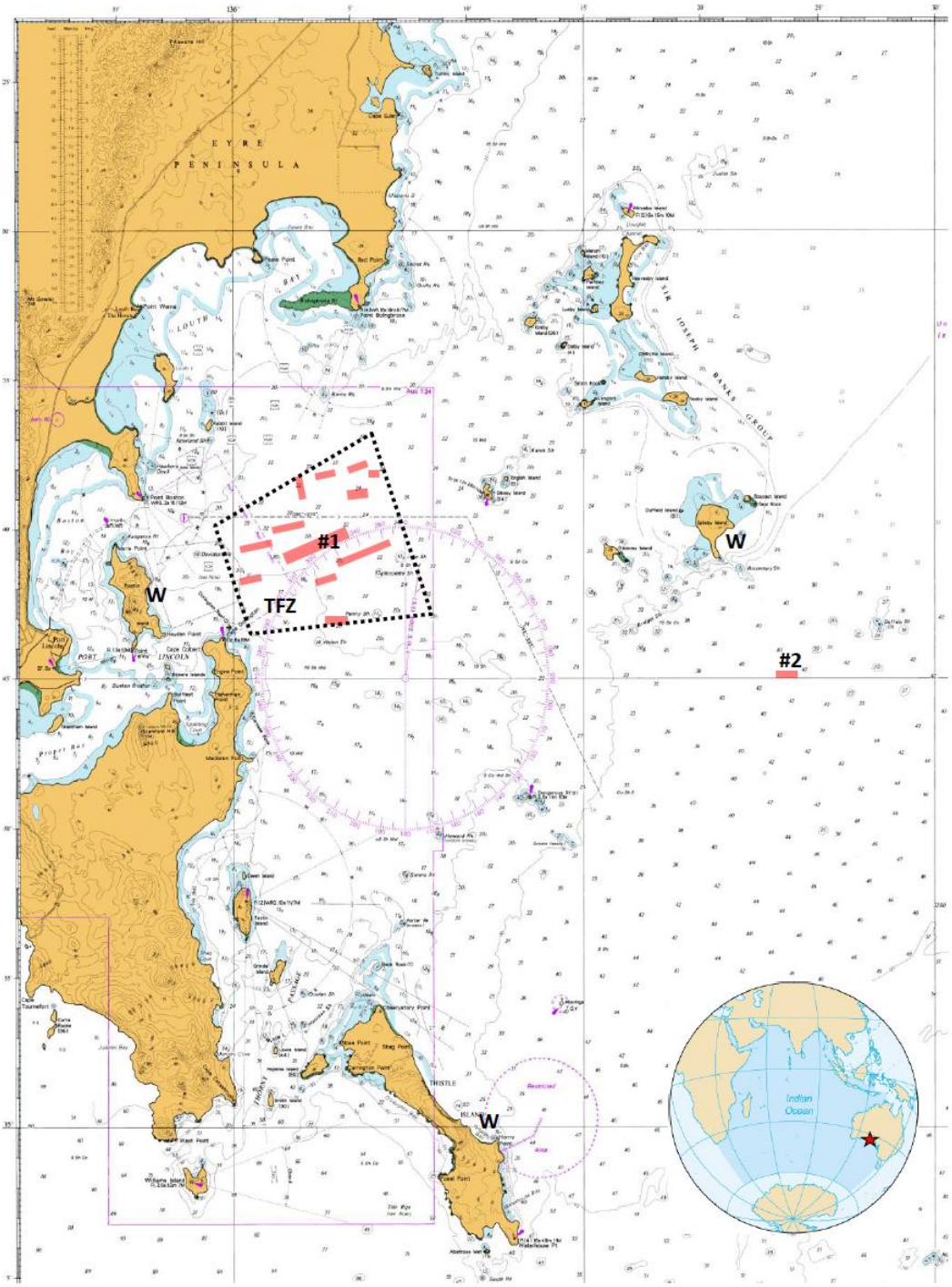

Figure 4.1 Map of Boston Bay in the south-west Spencer Gulf indicating commercial lease sites in red. Also noted are the near shore cage study site (#1) and offshore cage site (#2), and the 2010 Tuna Farming Zone in dotted box. 'W' denotes weather stations used for wind speed measurements.

SBT were fed frozen sardines at an average rate of 0.8 kg SBT^{-1} day^{-1} for their entire ranching period. In 2010, commercial sites within the near shore TFZ ranged in size between 63 and 341ha, with an average biomass at harvest of 2,283kg ha^{-1}. The whole near shore TFZ is 17200ha, of which 1569.5ha was under commercial lease in 2010. The commercial offshore site was 100ha, with an average biomass at harvest of 3,087 kg ha^{-1}. In 2010 the whole offshore site was 38350ha.

4.2.2 Field Collection

Ranched SBT are wild and each cohort consists of several schools of SBT mixed together, therefore individual SBT were used as replicates for this study, not cages of SBT, to measure the effect of ranching site on SBT. Sampling was limited due to the high commercial value of the fish, limiting total sample size to 100 SBT. Three sampling time points of samples were chosen for this study: at transfer into grow out cages to demonstrate initial differences between the cohorts of fish, at week 6 of ranching duration to demonstrate effects of site on ranching performance and at week 23 to demonstrate effects of site on long term ranching performance. Week 6 was chosen as the most important sampling time point for two reasons: (1) limited effects of captivity on ranched SBT prior to 6 weeks have been observed and (2) a significant mortality event and health changes are known to occur at week 6 in near shore ranched SBT (Kirchhoff *et al.* 2011a). Samples were collected from both the near shore and offshore SBT at transfer (n=10 per site), week 6 (n=20 per site) and week 23 at harvest (n=20 per site) post transfer. Transfer samples were collected during transfer from the tow cage to the grow-out cage and the week 6 and week 23 samples were collected during commercial harvests. At the initial and week 6 time points, SBT were sampled using a baited hook and line. Divers caught the SBT at the 23 week sampling. The total time between capture and killing of each SBT was less than one minute for both catching methods. Once on the boat, SBT were immediately spiked in the head, the brain was removed using a 'Taniguchi tool' (core), and a wire was placed down the spine to destroy the spinal nerves. Length and weight was recorded for all SBT at the time of sampling. At transfer and week 6, SBT were weighed whole, but at week 23 SBT were weighed after the gills and viscera were removed due to space limitations associated with large commercial harvests. Weight for SBT sampled in week 23 was corrected by dividing weight (kg) by 0.87 [9]. Condition index was calculated for each sample using the formula: weight (kg) divided by length $(m)^3$. Immediately after external surface examination, whole blood was collected from the severed

lateral artery in the pectoral recess in two 10ml tube (Sarstedt, Ingle Farm, South Australia), one heparinized and one non-heparinized, and placed on ice. Blood was collected within 3 minutes of fish capture. During transfer and week 6 sampling, parasites were quantified. External metazoan parasites were quantified from both the skin and gill arches using the naked eye during killing or as soon as possible after the SBT were killed. All lice visible to the naked eye were collected as soon as possible; any additional lice remaining on tuna surfaces were then detected using a technique described previously (Hayward *et al.* 2010). Parasites were not quantified from the week 23 samples as previous studies have determined parasites loads to peak on ranched SBT earlier in the ranching season (Hayward *et al.* 2009). The gills and viscera were then excised. The heart was placed in a waterproof tub, the visceral organs were placed in a waterproof bag and both stored on ice.

4.2.3 Laboratory Processing

The heparinized vial of whole blood was used for whole blood and plasma aliquots. Three 500µl aliquots of whole blood were transferred into 1.5ml plastic tubes and frozen at -20°C. The remaining blood was centrifuged at 3000xg at 4°C for 5 minutes. Blood plasma was aliquoted into five-1.5ml plastic tubes, and frozen at -20°C. The non-heparinized vial of whole blood was used for serum collection. Vials were stored upright at 4°C for 24 hours, centrifuged at 1000xg at 4°C for 5 minutes, and serum aliquoted into three 1.5ml tubes. Serum samples were stored at -20°C. Hearts were dissected 2–4 h after removal from the carcass and flushed with physiological saline to dislodge any adult *Cardicola forsteri* (see Aiken *et al.* 2006). Flushes were then poured into Petri dishes and examined for the presence of adult *C. forsteri* using a dissecting microscope.

4.2.3.1 Blood Variables: Hematology

Hemaoglobin concentrations were determined from whole blood aliquots using the cyanometahemaoglobin assay based on Brown (1984) modified by Kirchhoff *et al.* (2011b).

Blood plasma glucose and lactate were measured using Accu-Chek ® Advantage II and Accutrend ® Plus by Cobas, respectively. The pH of blood plasma samples was measured using a Minilab Isfet pH

meter Model IQ125 (IQ Scientific, USA). Blood plasma osmolality was determined using a Vapro© Model 5520 vapour pressure osmometer (Wescor Inc., Logan, Utah, USA).

4.2.3.2 Blood Variables: Humoral Immune Response

Blood serum was analyzed in triplicate for lysozyme activity and alternative complement activity. Lysozyme activity was measured using a method based on that described by Carrington & Secombes (2007) modified by Kirchhoff *et al.* (2011b). Blood serum alternative complement activity was measured using a modified Yano (1992) method as described by Kirchhoff *et al.* (2011b).

4.2.4 Statistical analyses

Parasite infections were characterized by prevalence (the number of host infections as a proportion of the population at risk), mean intensity (the average number of parasites per infected host) and mean abundance (the average number of parasites in all hosts) (Bush *et al.* 1997). Sterne's exact 95% confidence intervals were calculated for prevalence, and 95% bootstrap confidence intervals (with 2000 replications) were calculated for mean abundance, using the software 'Quantitative Parasitology 3.0', supplied by Reiczigel and Rózsa (2005). The prevalence and mean abundance for each species were compared between treatments and ranching durations in a pairwise fashion. Given the high total number of pairwise comparisons, $\alpha = 0.01$ was regarded as significant for these statistics.

All other performance, haematology, and immunology results were interpreted using the R 2.8.1 statistical package (© 2008, The R Foundation for Statistical Computing). Survival was assessed using log-rank test for equality of the two Kaplan-Meier survival curves, one for each treatment. Condition index and blood parameters were analyzed for differences between each treatment at each sample date, and differences within a treatment for all sample dates using ANOVA. The assumption of homogeneity of variances was checked by the residual plot and Bartlett test and variables transformed when necessary. The Tukey HSD post-hoc test was applied at a significance level of $\alpha=0.05$, to determine differences between the explanatory variables. Plasma pH was log 10 transformed due to failure to pass the Bartlett test for normalcy.

4.3 Results

While the offshore cohort began ranching (i.e. at transfer) with a lower condition index ($F=5.7614$, $df=1,18$, $p=0.0274$), their condition increased considerably between transfer and week 6 of ranching (Table 4.2). At week 6, the offshore cohort averaged higher condition index compared to the near shore cohort ($F=5.5738$, $df=1,38$, $p=0.0235$). The offshore cohort maintained condition index from week 6 to week 23 of ranching ($p>0.05$), while the near shore cohort continued to increase to a condition equal to the offshore cohort by week 23 ($F=0.569$, $df=1,37$, $p=0.4554$). Changes in condition index can be expected to occur in an asymptotic fashion during fattening, quickly increasing from low to medium conditions and increasing much slower at higher condition.

The offshore cohort had higher survival through the ranching period ($\chi^2= 107$, $df=1$, $p<0.001$) (Figure 4.2), with at 5.6% cumulative mortality compared to 10% of the near shore cohort. Initial mortality was higher in the offshore cohort, and may be attributed to different conditions on the tow from the capture site to the lease site or due to initial unfavorable conditions at the offshore lease site early in the ranching season. Nearly 80% of the total mortality in the offshore cage occurred in week 1 and 2 of ranching. The near shore cohort had little initial mortality, with 84% of the total mortality occurring between week 8 and 12 of ranching. An approximate 100 SBT were unaccounted for in the weekly mortality counts for the offshore cohort. Upon consultation with the farm manager, it was assumed these fish were victim to either shark attacks or poaching, although neither assumption can be confirmed. Cumulative mortality in the offshore cohort was 2.5% when the unaccounted SBT were not included in the calculation.

At week 23 of ranching, the offshore cohort had 1.5g dL^{-1} higher hemaoglobin concentration ($F=15.920$, $df=1,38$, $p<0.001$) and 20mmol kg^{-1} higher osmolality ($F=9.7547$, $df=1,38$, $p=0.003$) compared to the near shore cohort (Table 4.2). While blood plasma lactate was higher in the offshore cohort at transfer ($F=20.592$, $df=1,18$, $p<0.001$), it was not different at week 6 or week 23 of ranching (Table 4.2). Therefore the initial difference may be attributed to differences between the cohort in tow and/or transfer conditions, not effects of location. No other differences were found in blood parameters or performance between cohorts ($p>0.05$) (Table 4.2).

Table 4.2 Mean ± SE for length (cm), weight (kg), condition index and blood parameters in ranched SBT at transfer, week 6 and week 23 of ranching in the near shore and off shore cohorts. Harvest weights are corrected for gg weight collection. Blood parameters include hemaoglobin (Hb) (g dL-1), plasma pH, plasma osmolality (mmol kg-1), plasma glucose (mmol L-1), plasma lactate (mmol L-1), lysozyme (ug mL-1), and alternative complement (ACH50) (units mL-1) activity. Different letters denote significant differences over time within each treatment. * denotes statistical differences between the offshore cohort and the near shore control cohort..

	Near shore			Offshore		
	Transfer	**6 weeks**	**23 weeks**	**Transfer**	**6 weeks**	**23 weeks**
Length	96.9±1.6	114.3±2.6	117.8±1.7	99.0±2.7	116.9±2.8	122.2±2.1
Weight	17.9±1.0	32.6±1.9	38.4±1.5	18.4±1.5	37.3±2.1	42.3±2.0
Condition Index	19.45±0.34^{a}	21.63±0.43^{b}	23.27±0.32^{c}	18.50±0.21^{a}*	23.08±0.44^{b}*	22.96±0.27^{b}
Hb	19.40±0.50^{ab}	20.33±0.37^{a}	18.84±0.27^{b}	18.95±0.43	19.59±0.50	20.12±0.34*
pH	7.87±0.05^{b}	8.09±0.03^{a}	7.66±0.03^{c}	7.96±0.05^{a}	8.04±0.02^{a}	7.62±0.03^{b}
Osmolality	394.2±4.5^{a}	412.6±4.5^{b}	451.5±4.6^{c}	388.4±4.5^{a}	421.4±5.1^{b}	477.5±6.9^{c}*
Glucose	3.03±0.29^{a}	5.79±0.35^{b}	6.22±0.16^{b}	3.65±0.74^{a}	6.39±0.37^{b}	6.58±0.27^{b}
Lactate	10.16±0.29^{ab}	11.09±0.39^{a}	9.59±0.29^{b}	6.72±0.70^{a}*	10.22±0.27^{c}	8.73±0.49^{b}
Lysozyme	71.69±27.56^{ab}	151.03±27.56^{a}	73.04±14.54^{b}	77.20±25.71^{a}	202.25±25.71^{b}	55.34±14.17^{a}
ACH50	184.38±23.81^{a}	119.56±10.30^{b}	85.28±6.08^{c}	236.54±65.91^{a}	141.79±14.75^{b}	82.35±13.03^{b}

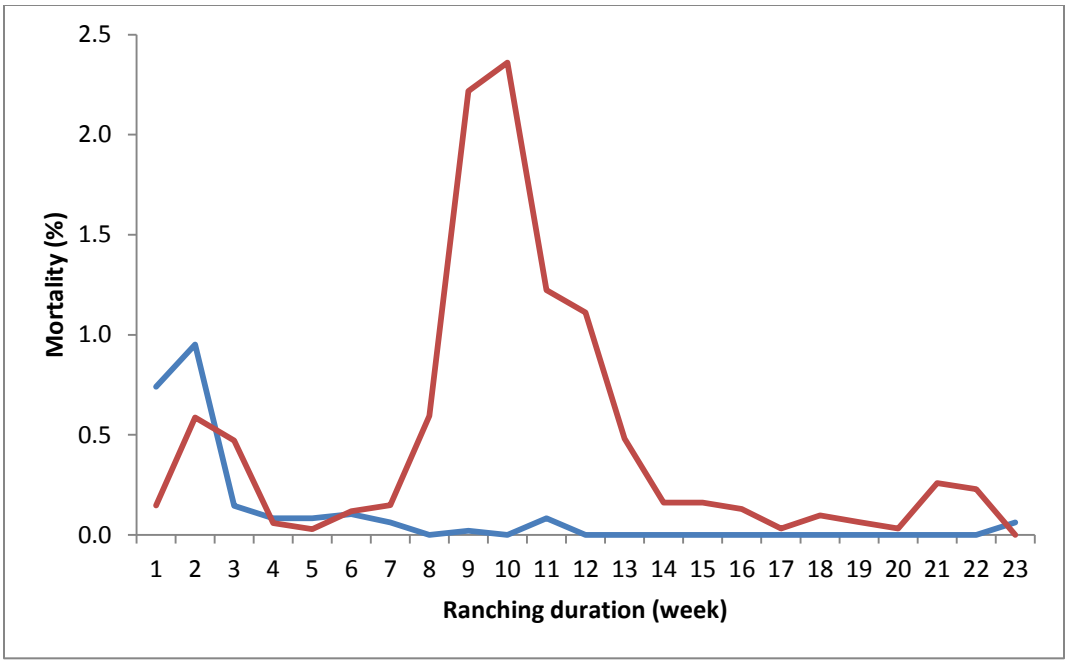

Figure 4.2 Weekly mortality in ranched SBT in the near shore (denoted in red) and off shore cohorts (denoted in blue). There was a difference in survival curves over the first twelve weeks of ranching ($\chi 2= 107$, df=1, $p<0.001$).

Offshore fish had lower prevalence (p=0.048) and mean abundance (t=-2.366, p=0.0235) of *Caligus* spp at 6 weeks of ranching compared to near shore SBT. While offshore SBT maintained low *Caligus* infections from transfer to week 6, the prevalence in the near shore cohort increased from 0 to 55% (p=0.004) and the mean abundance increased from 0 to 0.65 *Caligus* per fish (t=3.901, p=0.0045) (Table 4.3). There was no *Cardicola forsteri* infection within the offshore cohort between transfer and week 6 of ranching. Prevalence of *C. forsteri* in the near shore cohort increased from 20 to 85% (p=0.001), mean intensity from 1 to 4.18 flukes (t=4.452, p=0.001), and mean abundance from 0.20 to 3.55 (t=4.741, p=0.001) over the same time period (Table 4.3). No differences in prevalence, mean intensity, or mean abundance of *Caligus* sp or *Cardicola forsteri* were found between the two sites at the start of ranching (i.e. at transfer). There was no effect of sampling date or location on the mean intensity of *Caligus* sp.. No differences were found in prevalence, mean intensity, or mean abundance of gill parasites (*Hexostoma thynni, Pseudocycnus appendiculatus,* and *Euryphorus brachypterus*) (Table 4.3).

Table 4.3 Parasite prevalence (P) (95% confidence interval), mean intensity (I) (95% confidence interval) and mean abundance (A) (95% confidence interval) in ranched SBT at transfer and week 6 of ranching in the near shore and off shore cohorts. n.a. denotes calculations which were not available. Different letters denote significant differences over time within each treatment. * denotes statistical differences between the offshore cohort and the near shore control cohort.

	Near shore					
	Transfer			Week 6		
	P (%)	I	A	P (%)	I	A
***Caligus* spp.**	0.0 (0.0-29.1)[b]	0.0 (na)	0.00 (0.00-0.00)[b]	55.0 (32.0-75.6)[a]	1.2 (1.0-1.6)	0.65(.35-1.00)[a]
Hexostoma	10.0 (.5-44.6)	1.0 (0.0-1.0)	0.10 (0.00-0.30)	15.0 (4.2-37.2)	2.0 (1.0-2.7)	0.30 (0.00-0.75)
Pseudocycnus	40.0 (15.0-70.9)	3.0 (1.5-5.0)	1.20 (0.30-1.80)	15.0 (4.2-37.2)	1.0 (0.0-1.0)	0.15 (0.00-0.30)
Euryphorus	20.0 (3.7-55.4)	2.5 (1.0-2.5)	0.50 (0.00-1.70)	25.0 (10.4-47.5)	1.2 (1.0-1.4)	0.30 (0.10-0.55)
Cardicola forsteri	20.0 (3.7-55.4)[b]	1.0 (na)[b]	0.20 (0.00-0.40)[b]	85.0 (62.8-95.8)[a]	4.2 (3.1-5.8)[a]	3.55 (2.35-4.95)[a]
	Offshore					
	Transfer			Week 6		
	P (%)	I	A	P (%)	I	A
***Caligus* spp.**	10.0 (0.5-44.6)	1.0 (0.0-1.0)	0.10 (0.00-0.30)	20 (7.1- 42.35)*	1.0 (0.0-1.0)	0.20 (0.05-0.35)*
Hexostoma	0.0 (0.0-29.1)	0.0 (na)	0.00 (0.00-0.00)	20.0 (7.1-42.4)	1.8 (1.0-2.5)	0.35 (0.10-1.05)
Pseudocycnus	20.0 (3.7-55.4)	4.0 (1.0-7.0)	0.80 (0.00-2.90)	5.0 (0.3-24.4)	2.0 (0.0-2.0)	0.10 (0.00-0.30)
Euryphorus	30.0 (8.7-61.9)	1.0 (0.0-1.0)	0.30 (0.00-0.50)	25.0 (10.4-47.5)	3.6 (1.8-5.4)	0.90 (0.25-2.05)
Cardicola forsteri	0.0 (0.0-29.1)	0.0 (n.a.)	0.00 (0.00-0.00)	0.0 (0.0-16.7)*	0.0 (n.a.)*	0.00 (0.00-0.00)*

4.4 Discussion

SBT maintained offshore had better survival, lower *Caricola forsteri* and *Caligus* parasite loads, and the hematology of SBT ranched offshore was equal to or exceeding SBT maintained in the traditional near shore ranching environment. These results suggest the offshore cohort may be able to respond better to ranching compared to SBT maintained near shore possibly due to better environmental conditions.

The observation of improved survival within the offshore cohort is the most significant outcome of this experiment. An average 6-14% cumulative mortality has been reported across the industry, occurring mostly in a restricted period from 6 to 12 weeks of ranching (Kirchhoff *et al.* 2011a). This annual mortality event has been observed within ranched SBT in South Australia since 1997 (ABSTIA pers. comm.) and was also observed within the near shore cohort of SBT in this study. The timing of this mortality, the duration of the event, and its severity is observed to vary annually (Nowak *et al.* 2009), between tows (Nowak *et al.* 2009, Kirchhoff *et al.* 2011b), by the timing of tow arrival within a season, between companies or husbandry techniques (Nowak *et al.* 2009), and even between cages within the same tow (Hayward *et al.* 2010, Kirchhoff *et al.* 2011b). Because the cause of the annual mortality event is unknown, it cannot be conclusively stated whether or not the offshore cohort maybe impacted in the future. Yet, current results suggest maintaining fish offshore may prohibit exposure of fish to the near shore mortality event, therefore maintaining enhanced survival in the future. A future study is currently underway to determine if temporary offshore holding can offer similar benefits to survival through to harvest.

The offshore cohort also demonstrated enhanced condition earlier in the season. Although the offshore cohort had a lower condition index at the beginning of ranching than the near shore cohort, they quickly gained in condition, both surpassing the near shore cohort and obtaining a condition equivalent to harvest quality by week 6 of ranching. The ability to have SBT reach harvest condition as early as possible in the ranching season is advantageous for the Australian commercial SBT operation, as it allows them to market fish earlier in the season for the fresh market. Not only does each SBT sold on the fresh market obtain a higher market value as opposed to the frozen market at the end of the season, but early stock

harvests reduce feeding and maintenance costs (ASBTIA pers comm.). The observed enhanced condition may be attributed to the lack of stress and an improved ability to convert feed into growth, and/or may demonstrate an improved ability to acclimate to ranching more quickly than those SBT maintained near shore.

Another promising result of offshore SBT ranching is a reduction in sealice and blood fluke infections. Described in 1997 (Cribb *et al.* 2000), a blood fluke, *Cardicola forsteri* is currently a common and prevalent infection in ranched SBT (Aiken *et al.* 2006, Hayward *et al.* 2010, Kirchhoff *et al.* 2011b), usually infecting up to 100% of ranched SBT after two months of captivity (Aiken *et al.* 2006). In 2004, *C. forsteri* was identified as one of the most significant risks associated with Australian ranched SBT (Nowak 2004), therefore reduction of this infection is an important result for the commercial industry. It is possible that the greater depth and current velocity may offer protection against infection by decreasing the incidence of cercariae within the cages as the intermediate host known to be a benthic terebellidae polycheate, *Longicarpus modestus* (Cribb *et al.* 2011). It may also be possible that the intermediate host is absent from the offshore site as its distribution is not known. Finally, enhanced health condition of the offshore SBT may also reduce infection success. Ranched SBT are able to develop a specific antibody response against *C. forsteri* and reduced infection burdens have been observed over ranching duration (Aiken *et al.* 2008). Research is currently underway to determine the trigger for specific antibody production to *C. forsteri* and its effects against current infection and future exposure. Further research is needed on the biology and distribution of the intermediate host and behavior and biology of the cercariae to assess a ranching site's risk for *C. forsteri* infection.

The lack of *Cardicola forsteri* infection observed within the offshore cohort provides a unique opportunity to investigate some of the claims of *C. forsteri* induced performance and health effects on SBT. It has been commonly assumed all ranched SBT maintained at the traditional near shore location are exposed to *C. forsteri* cercariae as soon as they enter the farming zone (Aiken *et al.* 2006). In the past researchers were not able to uncouple the effects of captivity from the effects of infection due to the 100% prevalence of *C. forsteri* within ranched SBT. It has been suggested *C. forsteri* infection may cause a reduction in hemaoglobin concentration (Hayward *et al.* 2010), an elevation in lysozyme concentration

(Hwayrd *et al.* 2009, 2010), and an elevation in alternative complement activity (Kirchhoff *et al.* 2011a). While there was no *C. forsteri* within the offshore cohort compared to 85% prevalence within the near shore cohort at week 6 of ranching, there was no difference between cohorts in hemaoglobin concentrations or humoral immune response. It is therefore unlikely that the observed mean intensity of infection with *C. forsteri* induces a hemaoglobin reduction or changes in humoral immune response, although infection intensities found in the near shore cohort were low and highly variable which may mask or prevent significant haematological differences. It may also be possible that the effect on hemaoglobin or humoral immune response to *C. forsteri* is short-lived (Kirchhoff *et al.* 2011a), therefore may have been missed by the large gaps between sampling times in this study. Lysozyme was significantly elevated in both cohorts at week 6, despite the absence of *C. forsteri* in the offshore group at this time. Complement activity progressively declined in both cohorts, despite the significant increase in *C. forsteri* prevalence and abundance in the near shore cohort and the absence of this parasite offshore. It has been suggested humoral immune response increases with duration of ranching (Watts *et al.* 2002), yet this trend was not observed within this study or within other previous studies (Kirchhoff *et al.* 2011a, 2011b). Previous research has found no association between *Cardicola forsteri* infections and performance of SBT, measured as condition index and mortality (Aiken *et al.* 2006). Yet, enhanced performance in the offshore cohort during the first few months of ranching may suggest a link which should be further investigated. There was also a reduction in mortality from week 6 to 12 of ranching, consistent with the suggestion that *C. forsteri infection* may be associated with mortality (Hayward *et al.* 2010, Kirchhoff *et al.* 2011a, 2011b). However, lower parasitic infections and reduced mortality in offshore SBT maybe a spurious relationship. Our results do not provide scientific evidence for the role of *C. forsteri* in SBT mortality or health effects due to the large number of differences between the offshore and the traditional near shore ranching environments and the limited number of sampling dates. However, this study does propose a potential role for offshore maintained SBT as a control group for future investigations into the effects of *C. forsteri.*

A lower infection of *Caligus* spp. was observed within the offshore cohort. An epizootic of *Caligus* spp. on ranched SBT is also a common and prevalent infection (Nowak 2004). Prevalence increased from 0% at transfer to 55% at week 6 in the near shore cohort, consistent with previous descriptions of ranched SBT

infection (Hayward *et al.* 2008, 2010). *Caligus* spp. prevalence has been shown to decline from week 6 onward so that by week 18 infection was largely absent from the ranching population (Hayward *et al.* 2008, 2009, Kirchhoff *et al.* 2011b). In contrast to *Caligus* infections in other farmed fishs, larval stages are rarely detected on ranched SBT, indicating Deagan's leatherjacket as an alternative source of mobile adult *Caligus* infections (Hayward *et al.* 2011). The reservoir of *Caligus*, Degen's leatherjacket (*Thamnaconus degeni*) (see Hayward *et al.* 2010), which are commonly attracted to the SBT grow-out cages during feeding (Hayward *et al.* 2008, 2009, Svane & Barnett 2008). These fish are benthic scavengers, and it has been suggested moving SBT into deeper water may reduce interactions between SBT and the source of the *Caligus*, therefore reducing infection rates (Svane and Barnett 2008). It is unknown if location differences alone can explain the decline in *Caligus* infection as husbandry differences may also be attributed. Enhanced feeding protocols may also reduce the attractiveness of the cages to opportunistic feeding by demersal fish. There is a relationship between the mean intensity of *Caligus* spp. infection and severity of eye damage as well as decreased condition index (Hayward *et al.* 2008, 2009, 2010). The offshore cohort had both reduced prevalence and abundance of *Caligus* spp. and enhanced performance, i.e. condition index, although a causal link between the two findings cannot be made. Again, no differences were found between near shore and offshore maintained fish in humoral immune response, suggesting no effect of *Caligus* infection. Although infection intensities observed within this study were low.

There were two further differences in haematology of the offshore cohort compared to the near shore cohort: osmolality and hemaoglobin concentration. Elevated osmolality was observed in offshore fish at week 23. Blood osmolality is known to increase when marine fish are not osmoregulating properly, for example at times of handling and transport (Redding & Schreck 1983, Urbinati *et al.* 2004, Gatica *et al.* 2010). During SBT end of ranching harvest procedures, fish are corralled into a restricted area, the increased fish density making diver associated harvest quicker and therefore more humane for the fish. Since no changes were observed in other stress-associated parameters of blood lactate and glucose concentrations, it is likely this corralling event just prior to harvest caused the elevated osmolality and not a long-term effect of ranching site. The offshore cohort was found to maintain stable hemaoglobin levels throughout the ranching season unlike the near shore cohort in which hemaoglobin concentration was

first elevated and then decreased between week 6 and week 23 of ranching. While the changes in hemaoglobin concentration observed within this study occurred at only one time point and the magnitude may seem physiologically insignificant, previous studies have determined changing hemaoglobin levels are associated with the mortality event in near shore fish (Kirchhoff *et al.* 2011a). Therefore the maintenance of stable hemaoglobin levels in the offshore fish may be further evidence these fish were not effect by the near shore mortality event and may be further evidence of better health and wellbeing of the offshore cohort compared to the near shore cohort.

Completing this study within the restrictions of commercial operations caused the experimental design to be unavoidably compromised in two ways: restricted sample size and two discrete cohorts were used for comparison. Sample size was maximized by limiting sample time points to those previously observed to yield the greatest significance. In addition, the effects of ranching site was discussed not only in comparison to fish ranched within the traditional near shore TFZ within the same season, but with historical data collected over several years. Although different cohorts of fish may react differently to ranching (Kirchhoff *et al.* 2011b), the parasite load, performance, and haematology observed in the offshore SBT was drastically different than expected variance between near shore maintained cohorts (Kirchhoff *et al.* 2011a, 2011b), therefore the importance of the findings within this study remain significant to the literature and our understanding of the effects of offshore finfish culture.

This is the first time the feasibility of offshore SBT ranching has been demonstrated on a commercial scale. A reduction in mortality and a reduction in the duration of fattening required to obtain harvest condition may outweigh the increased operation costs, therefore making the move offshore economically viable. In addition, numerous benefits of offshore culture including reduced blood fluke and sealice loads and haematological health equal to or exceeding near shore maintained fish may validate moving further offshore from an animal welfare point-of-view.

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CHAPTER 5:

CORRELATION OF HUMORAL IMMUNE RESPONSE IN SOUTHERN BLUEFIN TUNA, *T. MACCOYII*, WITH INFECTION STAGE OF THE BLOOD FLUKE, *CARDICOLA FORSTERI*

Kirchhoff, N.T., Leef, M.J., Valdenegro, C., Hayward, C.J., Nowak, B.F., (2012). Correlation of humoral immune response in southern bluefin tuna, *T. Maccoyii*, with infection stage of blood fluke, *Cardicola forsteri*. PLoS ONE, submitted.

Abstract

The blood fluke, *Cardicola forsteri*, is a prevalent infection in ranched southern bluefin tuna. This project aimed to define the timing and intensity of the various developmental stages of *C. forsteri* within southern bluefin tuna as well as to relate infection to host pathology and immune response. Archival samples from several cohorts of *T. maccoyii* sampled from 2008 to 2010 were used in this study. The prevalence and intensity of *C. forsteri* infection was described using heart flushes and histological examination. Humoral immune response, i.e. *C. forsteri* specific antibody, lysozyme activity, and alternative complement activity, was also described. Based on the validated and detailed *C. forsteri* infection timeline, relationships between infection events, physiological response, and diagnosis were proposed. Immune response developed concurrently with *C. forsteri* infection, with the majority of physiological response coinciding with commencing egg production. Further research is needed to confirm the origin of *C. forsteri* antigen which is responsible for immune response development and how *T. maccoyii* immune response works against infection. To aide this research, further diagnostic methods for confirmation of infection need to be developed.

5.1 Introduction

Understanding the physiological effects and immune response to blood fluke infection is not only important in assessing its economic impact on the southern bluefin tuna, *Thunnus maccoyii*, industry, but also in developing treatments to reduce infections or, potentially, to prevent infections all together. Like other blood flukes, *Cardiola forsteri* has a two-host lifecycle, which requires a definitive host, bluefin tuna, *Thunnus* spp., and an intermediate host, a marine polychaete *Longicarpus modestus* (see Cribb *et al.* 2011). Identified in ranched *T. maccoyii*, in 1997 (Cribb *et al.* 2000), *C. forsteri* has been found to infect SBT off the coast of Australia (Cribb *et al.* 2000; Colquittt *et al.* 2001; Munday *et al.* 2003; Deveney *et al.* 2005) and Atlantic bluefin tuna *T. thunnus* off the United States of America (Bullard & Overstreet 2004), off the coast of Spain (Aiken *et al.* 2007) and in the Adriatic Sea (Mladineo & Tudor 2004). Prevalence and intensity of *C. forsteri* infection increase rapidly during ranching (Aiken *et al.* 2006), with both the definitive and intermediate host present within the Tuna Offshore Farming Zone of Port Lincoln, South Australia (Cribb *et al.* 2011). Currently, the only pathological signs of infection have been limited to histological changes within the heart and gills (Colquittt 1999). No correlation has been found between infection and host condition, or physiological changes in plasma glucose, lactate, pH or osmolality (Kirchhoff *et al.* 2011a). A significant negative correlation with hemaoglobin concentration and positive correlation with humoral immune response, i.e. lysozyme, was reported in a rare case of hyperinfection (Hayward *et al.* 2010). However, these physiological effects may only be associated with hyperinfection, as these correlations were not found in a study which compared uninfected and infected SBT (Kirchhoff *et al.* 2011b). Furthermore, heavy infections of *C. forsteri* are rarely observed in ranched SBT (Nowak *et al.* 2006, Hayward *et al.* 2010). This is thought to be a consequence of host immune response (Watts *et al.* 2002). A specific antibody response to *C. forsteri* in August 2005 was positively related to the mean abundance of adult flukes in May 2005 in fish in the same pontoon, suggesting a delay between the infection and antibody response (Aiken *et al.* 2008). Currently, we do not understand what triggers anti*Cardicola* antibody activity to develop and how it acts against *C. forsteri*.

Understanding the timing and intensity of the various developmental stages of *C. forsteri* within SBT, may allow further understanding of host response to infection. First, the current infection timeline, inferred by a stochastic model based on size and reproductive status of adult flukes and biological data inferred from

other fluke species (Aiken *et al.* 2009), needs to be validated. Although, *C. forsteri* eggs have been observed within the spongy layer of the ventricle (Colquittt 1999, Colquittt *et al.* 2001), in the afferent filamental arteries and in the secondary lamellae of the gills (Colquittt *et al.* 2001), they have not been quantified nor the timing of their arrival within various organs has been described. The aims of this study were: (1.) to describe the timing of the appearance of the *C. forsteri* egg stage, (2.) to determine the timing of specific *C. forsteri* antibody activity development, and (3.) to correlate humoral immune response, i.e. lysozyme, alternative complement, and anti*Cardicola* antibody activity with the developmental stages of *C. forsteri* within SBT.

5.2 Material and Methods

5.2.1 Experimental Fish and Study Design

Archival samples from several cohorts of *T. maccoyii* sampled from 2008 to 2010 were used in this study (Table 5.1). Each tow of fish, which comprises of several schools of wild 2-4 year old *T. maccoyii*, is defined as a discreet cohort. $Cohort_{2009A}$ and $Cohort_{2009B}$ included samples which ranged from week 0, or at transfer into ranching pontoons, to harvest, at 19 weeks of ranching, and were used to assess long term effects as well as intra-annual variation. $Cohort_{2010}$ contained weekly samples which ranged from 4 to 9 weeks of ranching and were used to more closely examine more closely the time period when 100% of *T. maccoyii* are expected to be infected and the adult flukes are expected to mature. Changes from the wild, previously believed to be largely uninfected SBT, were examined by comparing $Cohort_{wild}$ to $Cohort_{2010}$ as these samples were collected in the same season. Inter-annual variation was examined by comparing $Cohort_{2009A}$ and $Cohort_{2009B}$ to $Cohort_{2010}$. Due to low infection intensities of adult flukes in 2009 and 2010, additional archival samples were obtained from $Cohort_{2008}$ which had comparatively higher mean intensity of adult *C. forsteri*. Unfortunately, histological samples were not available from $Cohort_{2008}$; therefore these samples could not be used for correlations of immune response with presence of *C. forsteri* eggs.

Table 5.1 *T. maccoyii* cohort characteristics. Several different types of samples were collected from each cohort: (Cf_a) *C.forsteri* adult fluke count within the heart, (Cf_{eg} *C.forsteri* egg count within the gills, (Cf_{eh}) *C.forsteri* egg and granuloma count within the heart, (B) blood serum. n.a. denotes data not available.

	Capture Date	Transfer Date	Stocking density (kg m^{-3})	length (cm)	weight (kg)	Sample type
$Cohort_{2008}$	17.02.2008	20.03.2008	0.19-2.58	95.06	23.14	Cf_a, B
$Cohort_{2009A}$	19.03.2009	28.03.2009	n.a.	95.40	16.28	Cf_a, Cf_{eg}, B
$Cohort_{2009B}$	23.03.2009	31.03.2009	n.a.	95.85	17.25	Cf_a, Cf_{eg}, B
$Cohort_{2010}$	06.12.2009	26.12.2009	2.376	92.45	15.80	Cf_a, Cf_{eh} Cf_{eg}, B
$Cohort_{Wild}$	21.01.2010	n.a.	n.a.	105.8	n.a.	Cf_a, B

All cohorts of SBT were captured using purse seine in the Great Australian Bight. All *T. maccoyii*, with the exception of the $Cohort_{wild}$ samples, were then towed to the Tuna Offshore Farming Zone of Port Lincoln, South Australia for commercial ranching. $Cohort_{wild}$ was captured within the Great Australian Bight during the capture of wild fish for ranching and sampled prior to towing SBT back to Port Lincoln. The cohorts were then transferred into grow-out pontoons and fed a diet of domestic and imported baitfish for their entire ranching period, approximately 3 to 6 months.

5.2.2 Sample collection

Each sampled *T. maccoyii* was captured using either a baited hook or by a commercial diver. Once landed on the boat, *T. maccoyii* were immediately spiked in the head, brain destroyed using a 'Taniguchi tool' (core) and a wire placed down the spine to destroy the upper spinal nerves. Total time between capture and killing of each *T. maccoyii* was less than 1 min. Whole blood was collected from the severed pectoral artery behind the pectoral recess in a 9 ml non-heparinized Vacutainer ® tubes (BD, USA) and placed on ice. Blood was collected within 3 min of fish capture. The heart was placed in a waterproof tub, the visceral organs were placed in a waterproof bag and both stored on ice. The whole blood was used for serum collection and vials were stored upright at 4°C for 24 h, centrifuged at 1000 xg at 4°C for 5 min, and serum aliquoted into three 1.5 ml tubes. Serum samples were stored at -20°C for up to one week and -80°C for long term storage.

Histological samples of the heart and gills were taken immediately upon returning to the laboratory, within 1-3 h of sample collection. Four 1 cm^3 sections of heart were obtained from the apex of the ventricle, each section including both the compact and spongy layer. The heart samples were fixed in 10% neutral buffered formalin for 24 h. In $Cohort_{2010}$ and $Cohort_{wild}$, three sections of gill lamellae were obtained from the second right gill arch: one at the each end and one from the centre of the gill arch. In $Cohort_{2009A}$ and $Cohort_{2009B}$, one section was obtained from the centre of the second right gill arch. Each section was excised from approximately halfway down the lamellae length. Histological examination from the middle of the second gill arch has been determined to be the most accurate for assessments of *C. forsteri* infection (Norte do Santos *et al.* 2012). In $Cohort_{2010}$ and $Cohort_{wild}$, the gills were fixed in seawater Davidson's fixative for 24 h. In $Cohort_{2009A}$ and $Cohort_{2009B}$, gills were fixed in 10% NBF for 24 h. All samples were then processed using standard histological techniques. After fixation, all tissues were cassetted and gills were decalcified for 1 h in Rapid Decalcifying Fluid (Australian Biostain, Australia). The heart and gill samples were then dehydrated in a graded ethanol series, cleared with xylene, embedded in paraffin, section at 5 µm and stained with haematoxylin and eosin. Histological sections of the gills were cut to maximize viewing of lamellae and secondary lamellae space where a majority of eggs were counted. This often came at the expense of viewing the filamental arteries, where a few eggs were observed.

5.2.3 Presence of *C. forsteri*

After histology sampling, hearts were dissected 2–4 h after removal from the carcass, and flushed with physiological saline to dislodge any adult *C. forsteri* (see Aiken *et al.* 2006). Flushes were then poured into Petri dishes and examined for the presence of adults using a dissecting microscope. $Cohort_{wild}$ hearts were stored frozen. They were thawed and then flushed with physiological saline and flushes examined as described above. Number of adult flukes was reported as a total number per infected fish.

C. forsteri egg counts were completed on all four heart sections and at least 2 of the 3 gill sections, when available. All sections were viewed under a light microscope using a 40x objective and any eggs within the section quantified. Eggs were identified using the description provided by Colquittt *et al.* (2001). An egg was counted if 100-50% of the internal integrity of the egg was intact. To describe the inflammatory

response associated with eggs, in $Cohort_{2010}$, eggs in the heart were further subdivided into those with or without an associated granuloma. An egg was considered one without a granuloma if there was little observed host response and 50-100% of internal integrity of the egg was intact. Eggs with granulomas were counted when a granuloma was visible, i.e.the egg was surrounded by a significant layer of host cells, and 20-100% of the internal integrity of the egg was intact. All histological slides were then scanned and analyzed with J-image version 1.45 to determine total surface area of the tissue. Egg counts were reported as number per cm^2 surface area of histological section examined.

5.2.4 Anti*Cardicola* antibody activity

Anti*Cardicola* antibody titre was measured following the method outlined by Aiken *et al.* (2008). Optimization of the method using checkerboard analysis required changes to the concentration and identity of reagents used in the ELISA and Western blot: an increase in blocker concentration from 0.3% to 1% casein-TBS, a decrease in rabbit anti-tuna heavy chain immunoglobulin (RATH) from 1:100 to 1:200, and a change from 1:8000 sheep anti-rabbit to 1:2000 goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma). Western blotting was used to investigate seropositive and seronegative samples according to the methods of Aiken *et al.* (2008). Different seropositive and seronegative samples than the Aiken *et al.* (2008) study were used as there was no access to the control samples from the previous study. The seropositive and seronegative controls were run on each plate. The dilution of each sample and control was converted into volume of serum within each well, and absorbance plotted against the log of serum volume. Titre, stated as antibody activity per volume of serum (units μl^{-1}) was calculated based on the equation from Arkoosh & Kaattari. (1990). A sample was defined as positive for anti*Cardicola* antibody activity when titre > 1 unit μl^{-1}.

The anti*Cardicola* antibody ELISA titration curve for the seropositive and seronegative controls was linear across the dilution range used in this study (Figure 5.1). At a 1:400 dilution, or 0.13 µl serum $well^{-1}$, the OD of the positive standard was 0.760±0.033 and the OD of the negative standard was 0.416±0.016. When each step was left out in sequence, background OD was <0.300 for 5 different plate observations.

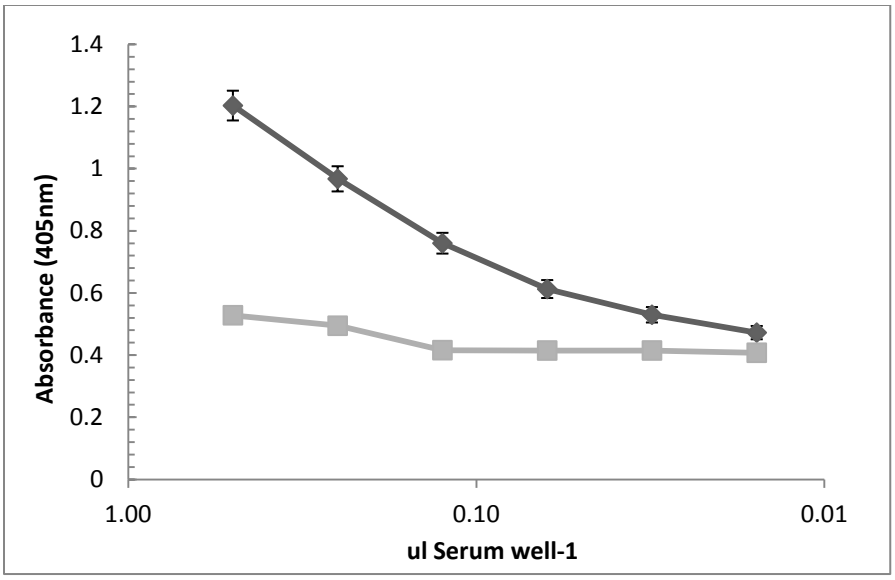


Figure 5.1 Anti*Cardicola* ELISA of positive (■) and negative (■) controls of *T. maccoyii* serum; mean ± SE of 15 observations.

5.2.5 Humoral immune response

Blood serum was analyzed in triplicate for lysozyme activity and alternative complement activity. Lysozyme activity was measured using a method based on Carrington & Secombes (2007) modified by Kirchhoff *et al.* (2011c). Blood serum alternative complement activity was measured using a modified Yano (1992) method as described by Kirchhoff *et al.* (2011c).

5.2.6 Statistics

Description of *C. forsteri* infection stages and humoral immune response as well as their correlations were interpreted using the R2.12.2 statistical package (©2011, The R Foundation for Statistical Computing). Numerous comparisons were also made between cohorts, as detailed in section 2.1. Each stage of *C. forsteri* infection was described by prevalence and intensity (Bush *et al.* 1997). Anti*Cardicola* antibody activity was described by prevalence (the number of hosts positive for immunoglobulin activity as a proportion of the total population). The prevalence was determined using a Sterne's exact method at N=1000 ± 95% confidence interval. Fisher's exact test was used to compare prevalence between cohorts and between sampling times. Mean intensity of *C. forsteri* infection was determined by bootstrapping at N=2000 ± 95% confidence interval. To examine changes in intensity and anti*Cardicola* antibody activity,

Kruskal Wallis, or Mann-Whitney-Wilcoxon test for pairwise comparisons, was used with a Bonferroni p-values correction. For two-variable comparisons between cohorts and time, *C. forsteri* intensity and anti*Cardicola* antibody activity had to be rank transformed prior to using a two-way ANOVA with Tukey's HSD post-hoc test. Humoral immune response was described using a two-way ANOVA followed by Tukey's HSD post-hoc test. Assumptions were checked by the residual plot, with lysozyme activity arcsine transformed and anti*Cardicola* antibody activity $\log_{10}+1$ transformed for all statistical analysis due to their failure to conform to homogeneity of variances. Spearman's two-sided correlation was used to compare humoral response to *C. forsteri* infection intensities. In correlations where anti*Cardicola* antibody activity was pooled over several sampling time points, each individual activity value was adjusted by subtracting the value of the mean activity level for the corresponding week of ranching, thereby removing the effect of time. Significance for all statistical analysis was assumed at $p \leq 0.05$.

5.3 Results

5.3.1 Characterization of *C. forsteri* infection

All life stages of *C. forsteri* within SBT increased in prevalence and intensity during ranching (Table 5.2). Adult *C. forsteri* infection was significantly greater during ranching compared to wild SBT, with both prevalence and intensity peaking several times within the traditional ranching duration (Table 5.2). The prevalence of adult *C. forsteri* increased from 0% in $Cohort_{wild}$ to 100% at week 5 of $Cohort_{2010}$ ($p<0.001$), with $Cohort_{2010}$ having higher prevalence than $Cohort_{wild}$ at all sampling time points ($p=0.003$). Both $Cohort_{2009A}$ and $Cohort_{2009B}$ increased from 0% prevalence at week 0, or the initiation of ranching, to ~100% at week 19, or harvest ($p<0.001$). The mean intensity of adult *C. forsteri* increased 4x from $Cohort_{wild}$ to week 5 of ranching in $Cohort_{2010}$. Within $Cohort_{2010}$, the intensity of infection decreased ($X^2=12.1327$, df=5, $p=0.033$) from 4.4 at week 5 to 1.5 at week 9. In $Cohort_{2009A}$ and $Cohort_{2009B}$ the mean intensity of adult *C. forsteri* increased approximately 4x from week 0 to week 8 & 19 of ranching ($Cohort_{2009A}$ $X^2=40.4442$, df=2, $p<0.001$; $Cohort_{2009B}$ $X^2=36.8906$, df=2, $p<0.001$). There was a significant amount of intra-annual variation in the prevalence of adult *C. forsteri* (week 8:$Cohort_{2009A}$ vs. $Cohort_{2009B}$,

p=0.048), although no differences were found for mean intensity. There was no evidence of inter-annual variation in prevalence or intensity of adult *C. forsteri*.

The prevalence and intensity of *C. forsteri* eggs within the heart increased significantly during ranching, also peaking numerous times within the traditional ranching duration (Table 5.2). *C. forsteri* eggs were primarily found within the spongiosa of the heart ventricles (Figure 5.2A&B), with only a few eggs being found in the compacta (Figure 5.2C&D). Eggs within the heart were observed with or without granulomas and at various stages of break-down. No miracidium development was observed within the heart. In gills, eggs were observed at the tips of the secondary lamellae, with only a few present within the secondary lamellae (Figure 5.2E). Eggs were observed at various stages of development within the gills, some with fully developed miracidia. No host response, such as granuloma, was observed in association with eggs within the lamellae or at the tips of the secondary lamellae. The prevalence of *C. forsteri* eggs within the heart increased from 0.45% in $Cohort_{wild}$ to 100% in $Cohort_{2010}$ by 7 weeks of ranching ($p<0.05$). In $Cohort_{2009A}$ & $Cohort_{2009B}$, the prevalence of eggs within the heart increased from ~31-35% at week 0 to 100% at week 19 ($p<0.001$). The mean intensity of eggs within the heart increased ~6x from the wild to week 7 of ranching ($X^2=5.8578$, df=0.016). Mean intensity of eggs within the heart increased from week 8 to week 19 in both $Cohort_{2009A}$ and $Cohort_{2009B}$ ($Cohort_{2009A}$ $X^2=43.8447$, df=2, $p<0.001$; $Cohort_{2009B}$ $X^2=39.6804$, df=2, $p<0.001$). In $Cohort_{2009A}$, mean intensity of eggs within the heart decreased from week 0 to week 8, in contrast to $Cohort_{2009B}$ which did not change. In $Cohort_{2010}$, the prevalence of egg associated granulomas in the heart increased at the same rate as the prevalence of eggs without associated granulomas, so that at any given time point approximately 50% or more of the eggs were associated with granulomas. The mean intensity of granulomas associated with *C. forsteri* eggs within the heart increased approximately 2x from week 4 and 5 of ranching to week 7, and an additional 5x from week 7 to week 8 and 9 of ranching ($X^2=34.658$, df=5, $p<0.001$) (Table 5.3). At harvest, a majority, if not all of the eggs in the heart were associated with granulomas in $Cohort_{2009A}$ and $Cohort_{2009B}$, although the eggs with and without granulomas were not quantified in the hearts of harvest fish due to time constraints. The maximum number of eggs found within the heart ventricle was 361.24 eggs cm^{-2} from $Cohort_{2009A}$ at harvest, or 19 weeks of ranching. There was significant intra-annual variation between cohorts in the prevalence and mean intensity of eggs within the heart (week 8: $Cohort_{2009A}$ vs. $Cohort_{2009B}$ prevalence

p=0.041 intensity χ^2=6.5926 df=1 p=0.010; week 19: $Cohort_{2009A}$ vs. $Cohort_{2009B}$ intensity χ^2=10.5366 df=1 p=0.001). There was significant inter-annual variation between $Cohort_{2009A}$ and $Cohort_{2009B}$ and $Cohort_{2010}$, with the prevalence of infection occurring approximately 1-2 weeks earlier in 2010 compared to 2009 ($Cohort_{2010}$ at week 8 vs $Cohort_{2009A}$ (p<0.001) and $Cohort_{2009B}$ (p=0.01) at week 8; prevalence $Cohort_{2010}$ at week 7 vs $Cohort_{2009A}$ (p<0.001) and $Cohort_{2009B}$ (p=0.01) at week 8; $Cohort_{2010}$ at week 9 vs $Cohort_{2009A}$ (p<0.078) and $Cohort_{2009B}$ (p=1.00) at week 8)There was no inter-annual variation in the mean intensity of eggs within the heart (p>0.01).

The prevalence and intensity of *C. forsteri* eggs within the gills increased significantly during ranching yet were patchy in their distribution and presence (Table 5.2). The prevalence of *C. forsteri* eggs in the gills increased from 0.91% in $Cohort_{wild}$ to 100% in $Cohort_{2010}$ at week 7, but then decreased to 0% at week 9 of ranching. There was no change in the prevalence of eggs within the gills in $Cohort_{2009A}$ over the ranching duration, with less than 30% of SBT positive with eggs in the gills at any sampling point, yet in $Cohort_{2009B}$ egg prevalence decreased from week 0 to week 19 (p<0.01). The mean intensity of infection increased ~100x between $Cohort_{wild}$ and week 7 of $Cohort_{2010}$ (X^2=5.6561, df=1, p=0.017). In $Cohort_{2009A}$ and $Cohort_{2009B}$, eggs were only found within the gills of SBT at week 19 of ranching at approximately 90 eggs cm^{-2} gill per infected SBT. The maximum infection intensity was 741 eggs cm^{-2} within $Cohort_{2010}$ at 7 weeks. There was no evidence of intra-annual or inter-annual variation in the prevalence and mean intensity of *C. forsteri* eggs within the gills. There was a positive correlation between the number of adult flukes and eggs within the heart (Spearman's rho= 0.550, df=184, p<0.001) (Figure 5.3A), and a negative correlation between the number of adult flukes and eggs within the gills (Spearman's rho= -0.283, df=175 , p<0.001) (Figure 5.3B) and between the number of eggs within the heart and gills (Spearman's rho= -0.387, df= 170, p<0.001) (Figure 5.3C) in $Cohorts_{2009A}$, $Cohort_{2009B}$, and $Cohort_{2010}$ combined.

Table 5.2 Description of *C. forsteri* infection by % Prevalence (P) (95% confidence interval) and mean intensity (I) (95% confidence interval) for adults within the heart, eggs within the heart and eggs within the gills of SBT. Significant letters denote statistical differences at $p<0.05$ within $Cohort_{2009A}$, $Cohort_{2009B}$ and between $Cohorts_{wild}$ & $Cohort_{2010}$. Intensity could not be directly compared statistically on samples with no infection present or with only one infected individual, denoted with a superscript n.a.

Cohort	Ranching Duration (week)	Adult Flukes			eggs cm^{-2} Heart			eggs cm^{-2} Gills		
		n	P	I	n	P	I	N	P	I
2009A	0 (transfer)	20	0.0 (0.00-37.71)^{c}	0.00 (NA)	16	31.3 (13.22-56.38)^{b}	6.60 (5.00-9.00)^{b}	19	0.0 (0.0-17.55)	0.00 (NA)
	8	20	70.0 (47.46-84.04)^{b}	4.07 (2.21-9.64)	20	15.0 (4.22-37.22)^{b}	2.67 (2.00-3.00)^{c}	20	0.5 (0.26-24.42)	1.00 (NA)
	19	19	100.0 (83.32-100.0)^{a}	4.8 (2.9-7.9)	20	100.0 (83.32- 100.0)^{a}	120.40 (92.15- 164.35)^{a}	20	25.0 (10.41-47.45)	90.4 (29.2- 201.2)
2009B	0 (transfer)	20	0.0 (0.0-16.68)^{c}	0.00 (NA)	20	35.0 (16.69-57.64)^{b}	5.00 (2.86-9.43)^{b}	19	0.0 (0.0-17.55)^{b}	0.00 (NA)
	8	20	45.0 (24.43-68.0)^{b}	3.33 (2.00-6.44)	20	50.0 (29.28-70.72)^{ab}	6.9 (4.40-11.10)^{b}	10	0.0 (0.0-29.08)^{ab}	0.00 (NA)
	19	20	95.0 (75.58- 99.74)^{a}	7.74 (5.32-11.84)	20	100.0 (83.32-100.0)^{a}	54.2 (37.90-79.00)^{a}	20	30.0 (13.96-53.54)^{a}	82.17 (22.00-196.50)
Wild	--	22	0.0 (0.0-15.7)^{b}	0.00 (NA)$^{n.a.}$	22	0.45 (0.24-22.21)^{b}	4.00 (NA)$^{n.a.}$	22	0.91 (0.16-29.07)^{c}	3.00 (1.00-3.00)^{b}
2010	4	8	87.5 (50.0-99.36)^{a}	3.14 (1.86-4.71)^{ab}	8	37.5 (11.12-71.07)^{b}	11.33 (9.00-12.67)^{a}	8	0.0 (0.0-36.46)^{c}	0.00 (NA)$^{n.a.}$
	5	6	100.0 (58.9-100.0)^{a}	4.40 (2.40-6.00)^{a}	6	66.7 (27.14-93.17)^{a}	6.50 (5.00-8.75)^{b}	6	83.3 (41.14-99.14)^{ab}	69.80 (1.80-194.2)^{ab}
	6	2	50.0 (2.5-97.5)^{a}	2.50 (2.00-2.50)^{ab}	2	50.0 (2.54-97.46)^{ab}	20.00 (NA)$^{n.a.}$	2	0.0 (0.0-77.63)^{abc}	0.00 (NA)$^{n.a.}$
	7	10	90.0 (55.36-99.48)^{a}	2.33 (1.56-3.00)^{ab}	10	100 (70.92-100)^{a}	23.10 (13.00-35.30)^{a}	9	100.0 (67.67-100)^{a}	329.00 (141.11-582.00)^{a}
	8	10	70.0 (38.1-91.3)^{a}	2.6 (1.60-4.00)^{ab}	10	100 (70.92-100)^{a}	9.60 (5.10-17.10)^{ab}	10	20.0 (3.68-55.35)^{bc}	8.00 (1.00-15.00)^{b}
	9	10	40.0 (15.01-70.91)^{a}	1.50 (1.00-1.75)^{b}	10	50.0 (22.25-77.75)^{a}	3.60 (1.20-5.80)^{b}	10	0.0 (0.0-29.08)^{c}	0.00 (NA)$^{n.a.}$

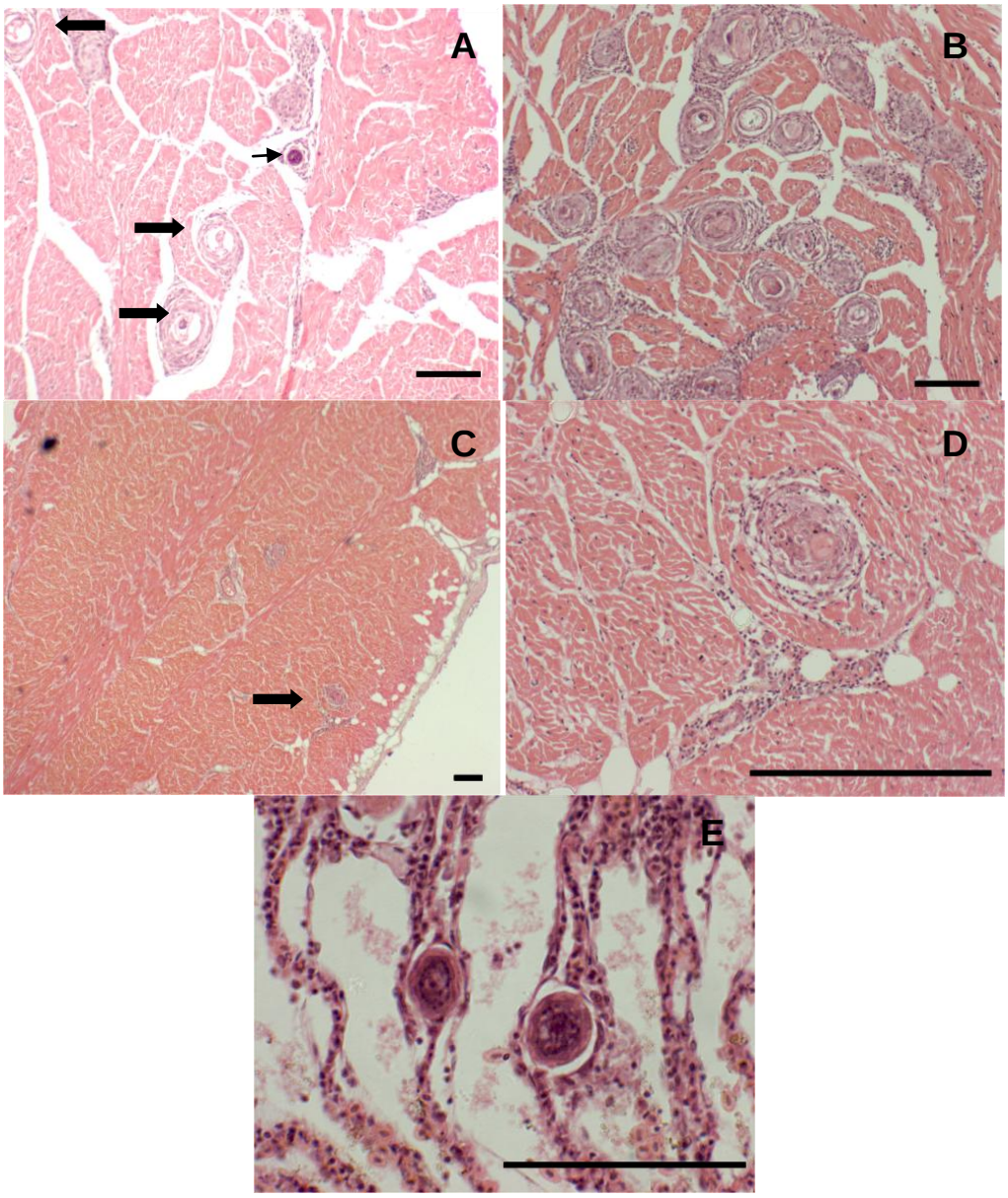


Figure 5.2 Presence of *C. forsteri* eggs in SBT organs H&E staining (A) within the spongiosa of the heart ventricles, eggs (small arrow) and granulomas (large arrow); (B) large number of granulomas associated with eggs within the spongiosa of the heart; (C & D) granuloma associated with eggs in the compacta of the heart ventricles (large arrow); (E) eggs within the lamellae creating a 'string of pearls' appearance. Scale bar 100µm.

Table 5.3 Presence of *C. forsteri* eggs in the heart of SBT with and without associated granulomas, % Prevalence (P) (95% confidence interval) and mean intensity (I) (95% confidence interval). Significant letters denote statistical differences at p<0.05. Intensity could not be directly compared statistically on samples with no infection present or with only one infected individual, denoted with a superscript n.a.

Ranching Duration (week)	n	Eggs without granulomas		Eggs with granulomas	
		P	I	P	I
4	8	25.0 (4.64-63.53)^{b}	1.50 (1.00-1.50)^{c}	37.5 (11.12-71.07)^{b}	12.33 (11.00-13.00)^{c}
5	6	50.0 (15.32-84.68)^{ab}	2.33 (1.00-3.33)^{bc}	66.7 (27.14-93.71)^{ab}	8.5 (7.25-9.25)^{d}
6	2	50.0 (2.54-97.46)^{ab}	2.00 (NA)$^{n.a.}$	50.0 (2.54-97.46)^{ab}	22.0 (NA)$^{n.a.}$
7	10	70.0 (38.06-91.27)^{ab}	5.14 (3.29-8.00)^{b}	100 (70.92-100)^{a}	26.70 (17.80-40.00)^{b}
8	10	100 (70.92-100)^{a}	85.2 (53.30-140.90)^{a}	100 (70.92-100)^{a}	94.80 (60.90-160.50)^{a}
9	10	100 (70.92-100)^{a}	123.50 (85.00-152.60)^{a}	100 (70.92-100)^{a}	125.40 (86.70-154.20)^{a}

5.3.2 Development of anti*Cardicola* antibody activity

Anti*Cardicola* antibody prevalence and intensity increased over ranching duration (Table 5.4). Anti*Cardicola* antibody activity was present in 31.8% of $Cohort_{wild}$ and increased to 100% by week 6 of ranching in $Cohort_{2010}$. At the start of ranching, week 0, 70% of $Cohort_{2009A}$ and 40% of $Cohort_{2009B}$, were positive for anti*Cardicola* antibody activity. In $Cohort_{2009A}$, the prevalence of anti*Cardicola* antibody activity increased 2x from week 8 to week 19. In $Cohort_{2009B}$, the prevalence of anti*Cardicola* antibody activity increased 2x from week 0 to week 8. At week 6, anti*Cardicola* antibody activity was ~6-44x higher in $Cohort_{2010}$ compared to $Cohort_{wild}$, (W=160.5, p<0.001). Anti*Cardicola* antibody activity peaked in $Cohort_{2010}$ at week 6 of ranching, decreasing by week 9 of ranching to an activity level still significantly higher compared to the wild (W=166.5, p=0.002). In $Cohort_{2009A}$, anti*Cardicola* antibody activity increased 3.7x between week 0 and week 19 of ranching (X2=24.51, df=2, p<0.001). In $Cohort_{2009B}$, anti*Cardicola* antibody activity increased 18x from the commencement of ranching to week 8 (X^2=21.0711, df=2, p<0.001). There was a significant intra-annual variation in anti*Cardicola* antibody activity over ranching duration when $Cohort_{2009A}$ was compared to $Cohort_{2009B}$ (F=22.4486, df=2,144, p<0.001). There was

significant inter-annual variation when $Cohort_{2010}$ was compared to $Cohort_{2009A}$ ($p<0.01$), but not when $Cohort_{2010}$ was compared to $Cohort_{2009B}$ ($p>0.05$) at week 8 of ranching. Therefore, it may be that the intra-annual variation in Cohorts is the most significant source of variation in anti*Cardicola* antibody activity.

5.3.3 Innate humoral response

Mean lysozyme activity was ~8-32x higher in $Cohort_{2010}$ compared to $Cohort_{wild}$ at week 5 ($F=9.9248$, $df=6, 59$, $p<0.001$). There was significant inter-annual variation (week 0, 8 & 19: $Cohort_{2010}$ vs. $Cohort_{2009A}$ & $Cohort_{2009B}$ $F=3.576$, $df=2, 47$, $p=0.0356$, but no evidence of intra-annual variation (week 0, 8 & 19: $Cohort_{2009A}$ and $Cohort_{2009B}$ $F=1.227$, $df=1, 95$, $p=0.271$).

Early during ranching alternative complement activity (ACH50) was lower in ranched compared to wild SBT, but it increased over the ranching duration (Table 5.5). ACH50 increased 2x from $Cohort_{wild}$ to $Cohort_{2010}$ at week 5 of ranching ($F=4.230$, $df=6, 59$, $p=0.001$). ACH50 decreased over ranching duration in $Cohort_{2009A}$ ($F=33.492$, $df=2, 57$, $p<0.001$) and $Cohort_{2009B}$ ($F=33.266$, $df=1, 38$, $p<0.001$). There was no evidence of intra-annual variation (week 8 & 19: $Cohort_{2009A}$ vs. $Cohort_{2009B}$ $F=0.3379$, $df=1, 95$, $p=0.5624$) or inter-annual variation in ACH50 (week 8: $Cohort_{2010}$ vs. $Cohort_{2009A}$ & $Cohort_{2009B}$ $F=0.4822$, $df=2, 45$, $p=0.6206$).

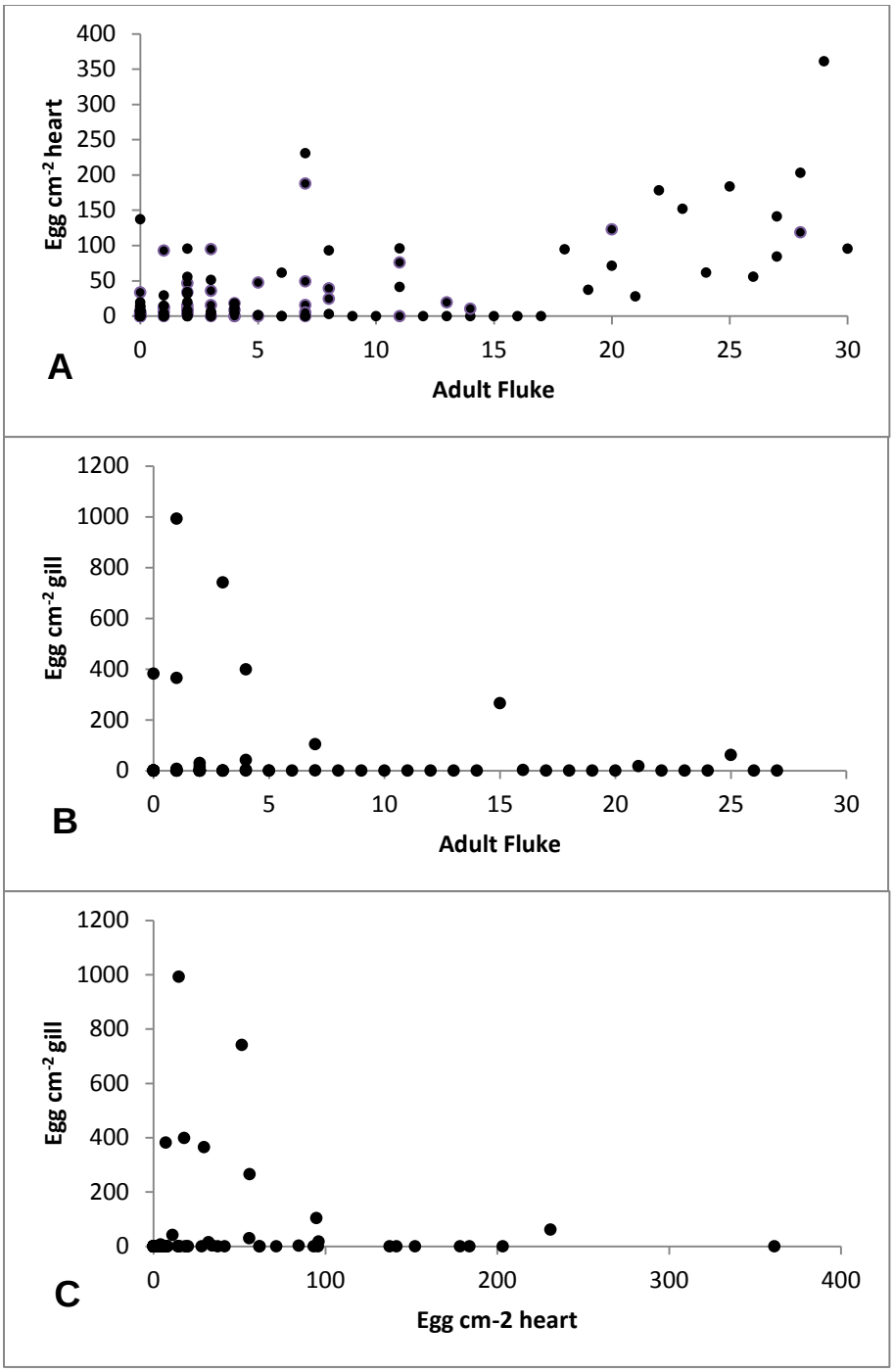


Figure 5.3 Correlation between (A) the number of adults and eggs in the heart, Spearman's rho= 0.550, df=184, p<0.001; (B) the number of adults and eggs in the gills, Spearman's rho= -0.283, df=175, p<0.001; (C) the number of eggs in the heart and eggs in the gills, Spearman's rho= -0.387, df=170, p<0.001 for $Cohort_{2009A}$, $Cohort_{2009B}$, and $Cohort_{2010}$ combined.

Table 5.4 Anti*Cardicola* antibody activity in SBT serum by prevalence (95% confidence interval) and mean activity ± SE. Significant letters denote statistical differences at $p<0.05$ within $Cohort_{2009A}$, $Cohort_{2009B}$ and between $Cohorts_{wild}$ & $Cohort_{2010}$.

Cohort	Ranching (week)	n	anti*Cardicola* Prevalence	anti*Cardicola* antibody activity units μl^{-1}
2009A	0 (transfer)	20	70.0 (47.46- 86.04)^{ab}	19.132 ± 7.886^{a}
	8	20	55.0 (32.00-75.57)^{b}	9.122 ± 3.640^{a}
	19	20	100 (83.32-100.00)^{a}	72.160 ± 11.095^{b}
2009B	0 (transfer)	20	40.0 (20.90- 62.77)^{b}	4.078 ± 1.570^{a}
	8	20	95.0 (75.58-99.74)^{a}	72.618 ± 19.551^{b}
	19	20	65.0 (42.36-83.31)^{ab}	23.364 ± 6.005^{a}
Wild	--	22	31.8 (15.18-54.65)^{b}	5.149 ± 2.902^{c}
2010	4	8	87.5 (50.00-99.36)^{ab}	64.833 ± 11.457^{ab}
	5	4	75.0 (24.87-98.2)^{ab}	86.978 ± 37.078^{ab}
	6	2	100 (22.37-100)^{ab}	222.817 ± 19.571^{a}
	7	10	100 (70.92-100)^{a}	99.327 ± 16.474^{ab}
	8	9	88.9 (55.66-99.43)^{a}	81.460 ± 32.010^{ab}
	9	9	88.9 (55.69-99.43)^{a}	34.918 ± 17.049^{b}

Table 5.5 Humoral immune response (mean ± SE or SD) in T. maccoyii. Significant letters denote statistical differences at p<0.05 within Cohort2009A, Cohort2009B and between Cohortswild & Cohort2010.

Cohort	Ranching (week)	n	Lysozyme Activity (µg ml^{-1})	n	ACH50 (units ml^{-1})
2009A	0 (transfer)	20	25.43 ± 8.99	20	156.40 ±18.63[a]
	8	20	171.88 ± 17.34	20	115.20 ± 12.30[b]
	19	20	33.49 ± 4.81	20	48.20 ± 4.10[c]
2009B	0 (transfer)	0	NA	0	NA
	8	20	83.74 ± 8.21	20	114.20 ± 7.37[a]
	19	20	96.08 ± 14.05	20	57.10 ± 6.70[b]
Wild	--	22	9.366 ± 2.071[c]	22	167.337 ± 16.626[b]
2010	4	8	144.143 ± 29.156[b]	8	241.918 ± 0.338[ab]
	5	4	295.975 ± 63.698[a]	4	330.0589 ± 0.526[a]
	6	2	198.200 ± 7.200[ab]	0	161.401 ± 41.028[ab]
	7	10	129.750 ± 34.779[b]	9	212.658 ± 21.926[ab]
	8	10	135.230 ± 33.057[b]	8	157.976 ± 29.173[b]
	9	10	110.490 ± 30.230[b]	10	150.481 ± 18.301[b]

5.3.4 Correlations between *C. forsteri* life stages and immune response

There was a negative correlation between the number of adult *C. forsteri* and anti*Cardicola* antibody activity in $Cohort_{2008}$ (Spearmans rho=-0.330, df=62, p=0.01), however the scatter plot shows this correlation was weak (Figure 5.4A). There was a positive correlation in $Cohort_{2010}$ at week 9 of ranching between the number of eggs within the heart and anti*Cardicola* antibody activity (Spearman's rho= 0.76667, df=10, p=0.02139) (Figure 5.4B).There were no other significant correlations in $Cohort_{2010}$ between anti*Cardicola* antibody activity and the number of adult flukes present, the number of eggs present in the heart, and the number of eggs present in the gills. There was no correlation in $Cohort_{2009A}$ and $Cohort_{2009B}$ between anti*Cardicola* antibody activity, the number of adult flukes or the number of eggs present within the heart, or the number of eggs present in the gills.

In $Cohort_{2009A,}$ there was a significant negative correlation between the number of adult flukes and lysozyme activity (Spearman's rho=0.2848, df=59, p=0.029) (Figure 5.5A) and between the number of eggs within the heart and lysozyme activity (Spearman's rho= -0.2839, df=56, p=0.034) (Figure 5.5B). In $Cohort_{2010}$, there was a significant negative correlation between lysozyme activity and eggs within the

heart (Spearman's rho= -0.1261, df=44, p=0.042) (Figure 5.5C), but no correlation with adult flukes was found. No correlations with lysozyme were found in $Cohort_{2009B}$.

In $Cohort_{2009A}$ and $Cohort_{2009B}$, there was a significant negative correlation between the number of adult flukes and ACH50 activity ($Cohort_{2009A}$: Spearman's rho= -0.6777, df=59 , p<0.001; $Cohort_{2009B}$: Spearman's rho= -0.4882, df=40, p=0.001) (Figure 5.6A) and between the number of eggs within the heart and ACH50 activity ($Cohort_{2009A}$: Spearman's rho= -0.7073, df=56, p<0.001; $Cohort_{2009B}$: Spearman's rho= -0.5439, df=40, p<0.001) (Figure 5.6B). Although there was a significant correlation between the number of eggs within the gills in $Cohort_{2009A}$ (Spearman's rho=-0.2504, df=59, p=0.056) and $Cohort_{2009B}$ with ACH50 activity (Spearman's rho= -0.3939, df=30, p=0.031), this correlation was weak and based on a very few positive samples, therefore may not be accurate (Figure 5.6C). In $Cohort_{2010}$, there was no correlation between ACH50 and the number of adult flukes, the number of eggs within the heart or the number of eggs within the gills.

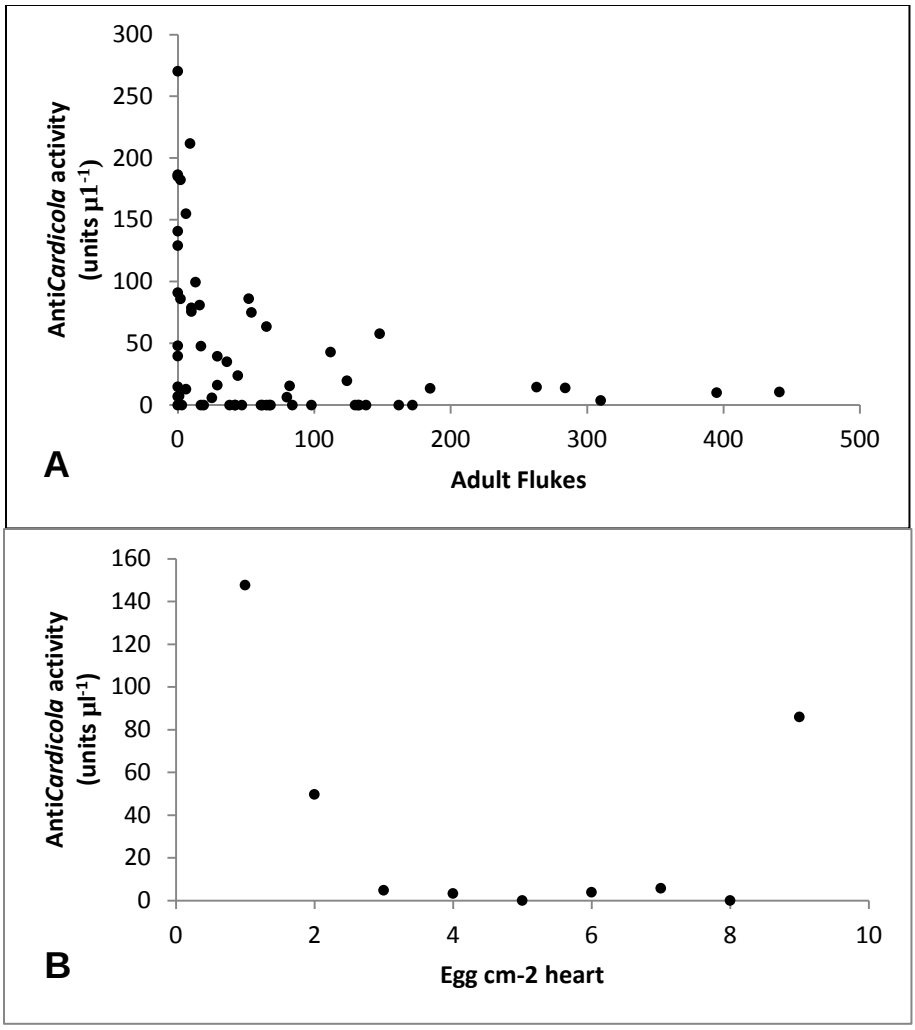


Figure 5.4 Correlations between Anti*Cardicola* activity in *T. maccoyii* serum and *C. forsteri*: (A) Cohort$_{2008}$, Spearman's rho= -0.330, df=62, p=0.01 (B) Cohort$_{2010}$ at week 9, Spearman's rho= 0.767, df=10, p=0.021

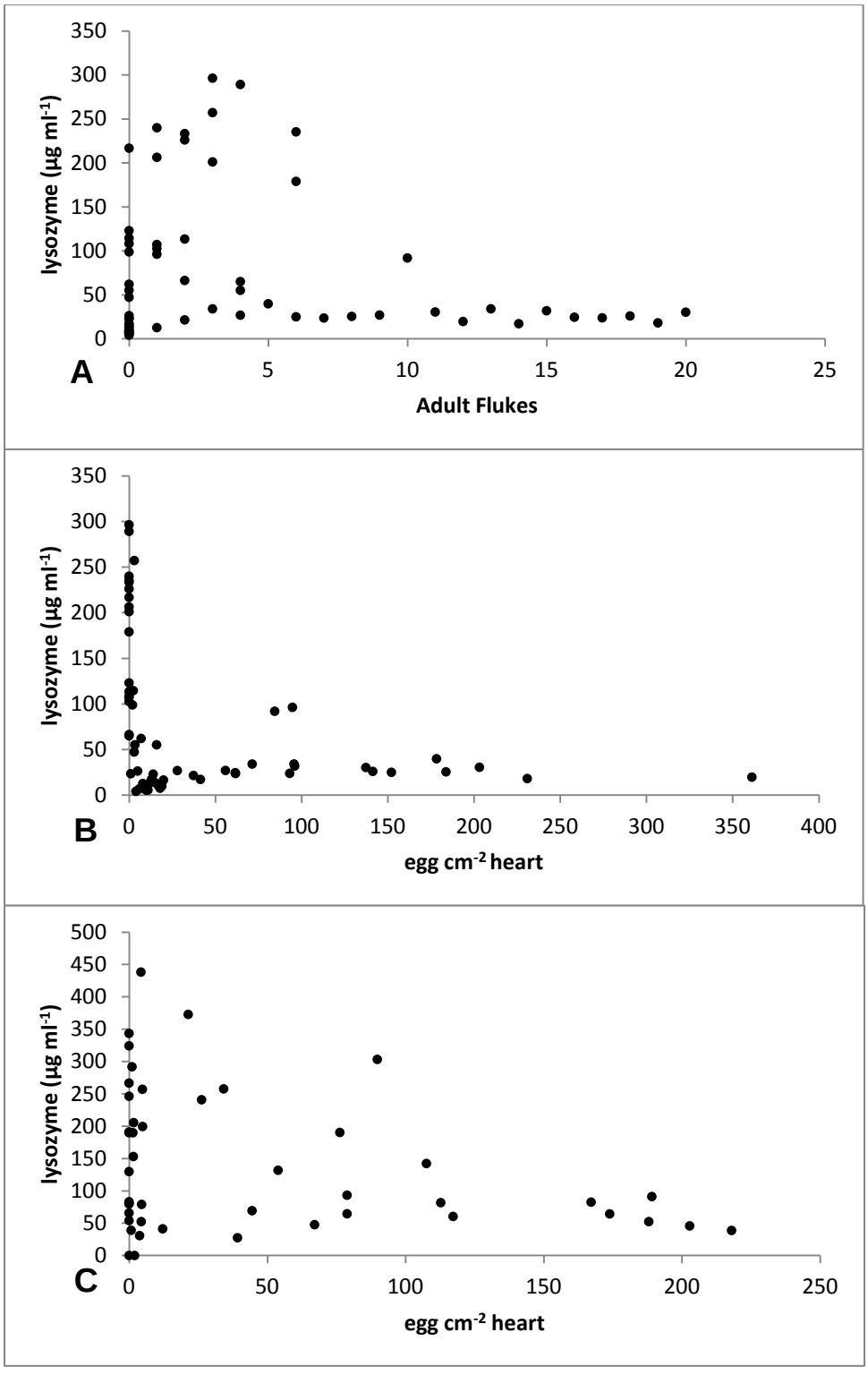


Figure 5.5 Correlation between lysozyme activity in *T. maccoyii* serum and *C. forsteri*: (A) Cohort$_{2009A}$, Spearman's rho= 0.2848, df=59, p=0.029; (B) Cohort$_{2009A}$,Spearman's rho=-0.2839, df=56, p=0.034;(C) Cohort$_{2010}$, Spearman's rho= -0.1261, df=44, p=0.042

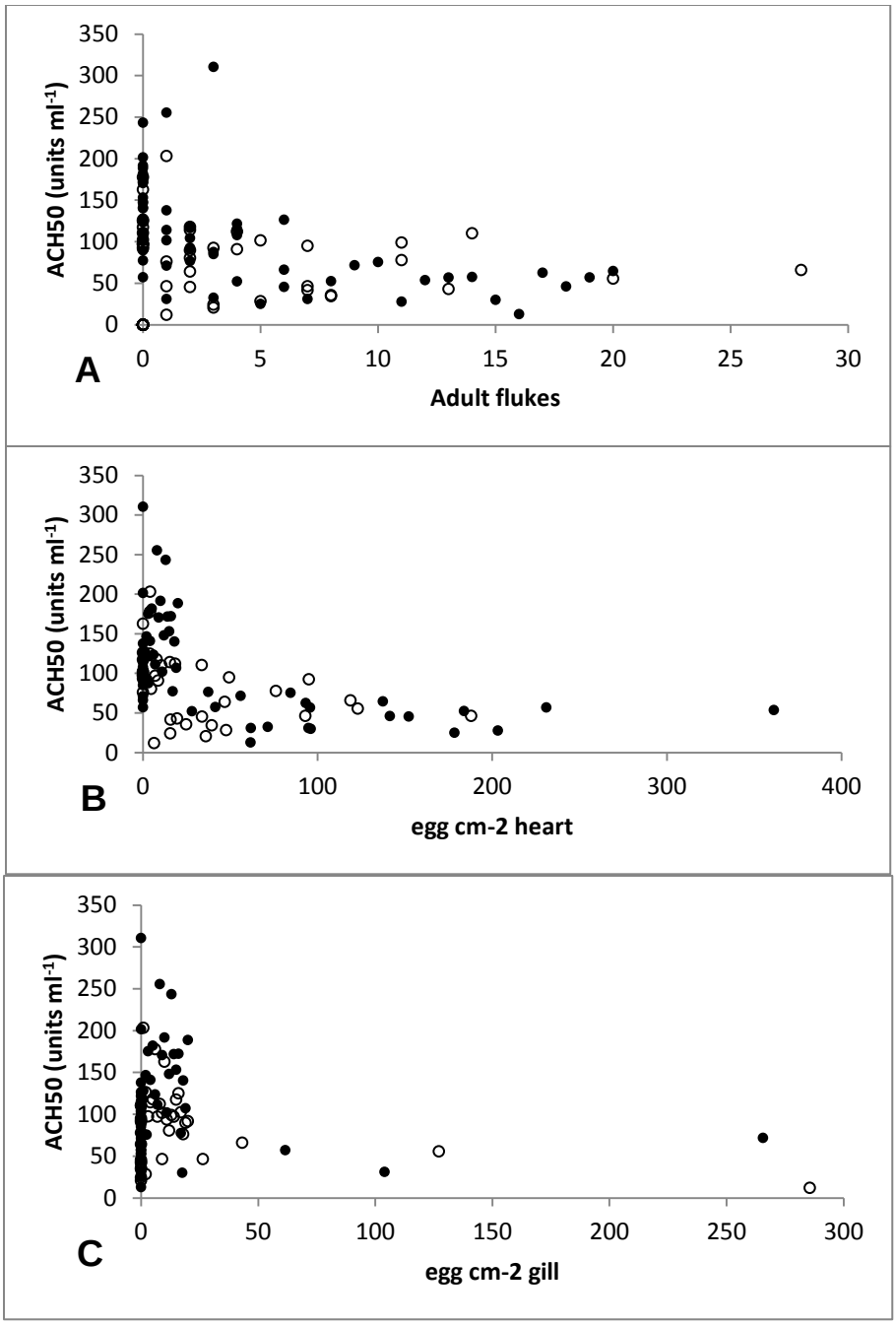


Figure 5.6 Correlations between *C. forsteri* infection and ACH50 activity in *T. maccoyii* serum from Cohort$_{2009A}$ (●)& Cohort$_{2009B}$ (○): (A) Cohort$_{2009A}$ Spearman's rho= -0.6777, df=59, p<0.001; Cohort$_{2009B}$ Spearman's rho= -0.4882, df=40, p=0.001; (B) Cohort$_{2009A}$ Spearman's rho= -0.7073, df=56, p<0.001; Cohort$_{2009B}$ Spearman's rho= -0.5439, df=40, p<0.001; (C) Cohort2009A Spearman's rho= -0.2504, df=59, p=0.056; Cohort$_{2009B}$ Spearman's rho= -0.3939. df=30, p=0.0311

5.4 Discussion

The prevalence and intensity of adult and eggs of *C. forsteri* increased with ranching duration, consistent with the previous reports (Aiken *et al.* 2006, Cribb *et al.* 2011). A majority of ranched *T. maccoyii* were infected by week 5 of ranching with eggs observed within the heart from week 4. The presence of eggs at this time is consistent with the previous assumed 27 day maturation (Aiken *et al.* 2009). This study observed one new location of *C. forsteri* eggs infection: compacta of the heart ventricles. All eggs within this study were assumed to be *C. forsteri* due to their appearance and confirmation by qPCR by Norte do Santos *et al.* (2012), although eggs observed within the heart need to be further confirmed to be *C. forsteri* as previous confirmation was completed on eggs within the gills only (Norte dos Santos *et al.* 2012). There was a strong positive correlation between the number of adults and the number of eggs within the heart and a negative correlation between the number of eggs within the heart and eggs within the gills for all Cohorts. These correlations validate the hypothesis adults release eggs from the heart and rely on the blood stream to transport eggs to the gills (Colquittt *et al.* 2001). Egg development was observed in the gills, but no development was observed in the heart; also consistent with Colquittt *et al.* (2001) observations. The observed egg infection intensity within the heart was consistent with Colquitt *et al.* (2001), who estimated ~19000 to 1.7 x 106 eggs within the heart of *C. forsteri* infected SBT. Accounting for the fish size differences, there were fewer eggs observed within the gills compared to a *Cardiola* spp. infection in Pacific Bluefin tuna, *Thunnus orientalis* (see Shirakashi *et al.* 2012). Intermittent changes in the intensity and prevalence of adult flukes over the ranching season, prolonged anti*Cardicola* antibody activity and the presence of eggs within the heart and gills much longer than the maximum estimated lifespan of *C. forsteri* (see Aiken *et al.* 2009), are consistent with the hypothesis SBT are continuously infected throughout the ranching season.

Immune response developed concurrently with *C. forsteri* infection progression, with a majority of immune changes occurring concurrent with commencing egg production. A peak in lysozyme and alternative complement activity coincides with fluke maturation. In carp infected by *S. inermis*, a similar peak and decline in complement activity was observed and believed to be related to the commencement of egg production (Roberts *et al.* 2005). Both lysozyme and alternative complement were negatively associated with adult and eggs at some time points, but not overall, which may suggest some parasite modulation of

immune system. This hypothesis was also suggested by Roberts *et al.* (2005) in explaining the humoral immune response in carp infected with *S. inermis*. Anti*Cardicola* antibody activity developed approximately a week after the peak in lysozyme and but a week earlier than alternative complement activity, and at a similar time as the production of eggs. Anti*Cardicola* antibody activity was present in the majority of *T. maccoyii* at approximately week 4 to 6 of ranching and remained at an elevated level for the remainder of the ranching season. Aiken *et al.* (2008) also found a gradual increase in antibody response through ranching season. No correlation was found between humoral immune response and eggs within the gills. Colquittt *et al.* (2001) believed lysozyme activity would increase when eggs are hatching from the gills, although an association with immune response may rely on a larger number of eggs within the gills than found in this study. In barramundi *Lates calcarifer* infected with *Cruoricola lates*, single miracidium hatching from the gills caused little damage, but when large numbers emerged, inflammatory response and haemorrhage occurred (Herbert *et al.* 1995). There was a negative correlation between the number of adults and number of eggs within the gills. The significance of this observation is unknown, but may be related to formation of granulomas trapping eggs within the heart and preventing their migration from the heart to the gills. A significant degree of intra-annual variation in infection and immune response was observed in this study. Previous studies also found annual variability in antibody levels (Aiken *et al.* 2008) and difference between the health status of various Cohorts of *T. maccoyii* (Kirchhoff *et al.* 2011c). The relationship between the health and immune status of *T. maccoyii* prior to the commencement of ranching needs to be investigated further as it may have significant effects on infection dynamics and success of treatment. The variation also complicates utilizing the natural infection model to infer physiological and immune response to infection.

The egg stage of infection may also be responsible for a majority of the physiological response. Although previously IHC confirmed *T. maccoyii* immunoglobulins did not to bind to eggs within the heart or gills (van Ede 2007), this study did find a positive correlation between anti*Caridicola* activity and the number of eggs within the heart. Anti*Cardicola* antibody activity correlated with eggs in the heart at week 9 of ranching, or approximately when granulomas begin to form, but not with eggs overall. The correlation between anti*Cardicola* antibody activity and eggs at the time of granuloma development, may suggest specific antibodies play a role in SBT immune response to *C. forsteri* eggs. However, the number of *C.*

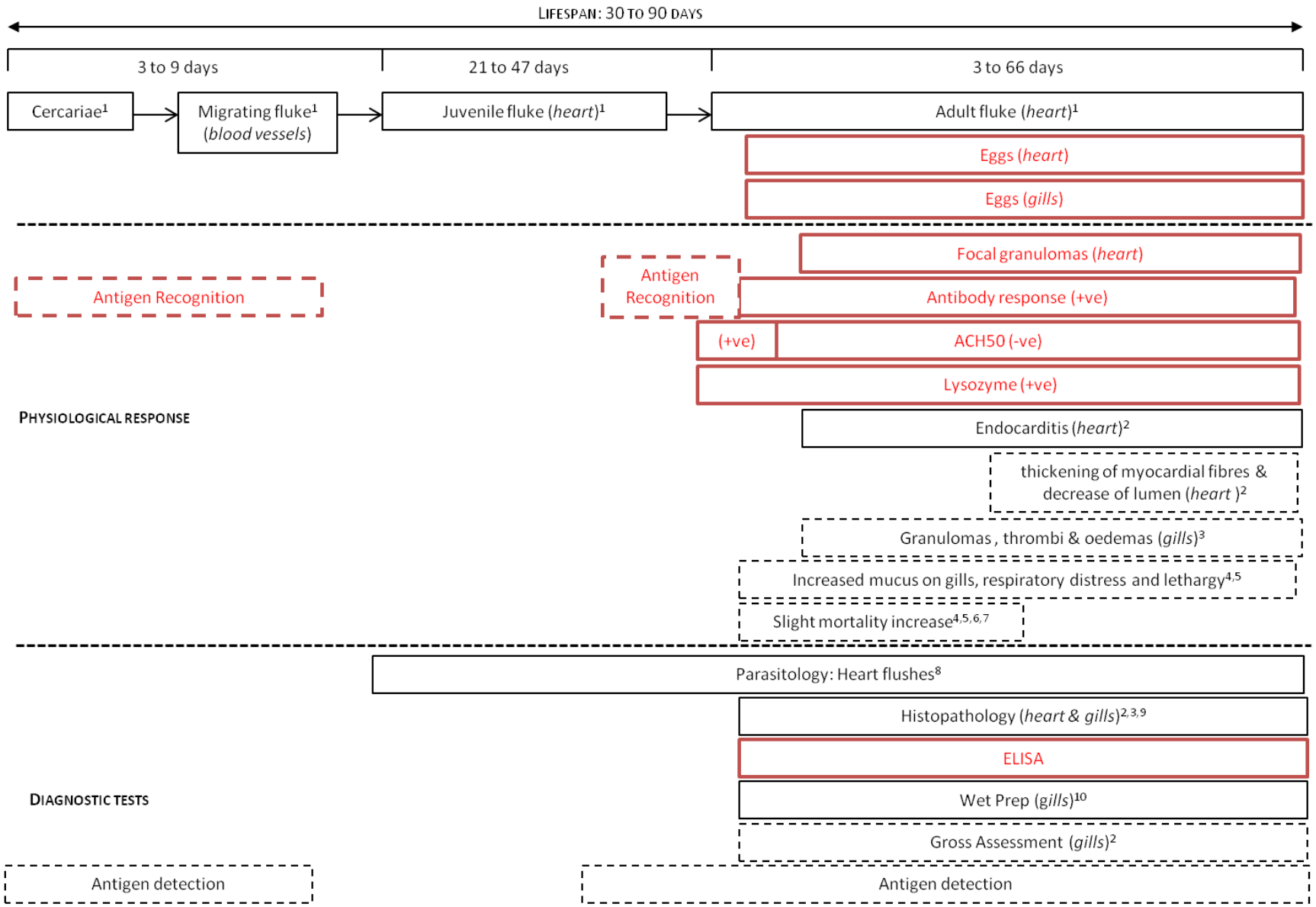
forsteri eggs continued to increase within the heart although immune response was developed. Aiken *et al.* (2008) proposed that *T. maccoyii* immune antibody response continued because of antigen from incoming *C. forsteri* larval stages. This is consistent with blood fluke infections in rats (Inbred Fischer/loc male rats) (see Smithers & Terry 1965). *T. maccoyii* immunoglobulins were found to bind to *C. forsteri* marginal spines and ventral tegument in IHC (van Ede 2007), therefore surface antigens on adult flukes may also promote immune response. Yet there was a weak correlation between anti*Cardicola* antibody activity and number of adult flukes within this study. As adult blood flukes live in host blood, they have evolved to avoid immune response in order to ensure parasite reproduction success (Cox 1997), therefore this finding was not unexpected. More research is needed to characterize *C. forsteri* antigens and which antigens are responsible for immune response development in *T. maccoyii*.

Based on these observations and previously published information (Colquittt *et al.* 2000, Rough 2000, Colquittt 2001, Munday *et al.* 2003, Aiken *et al.* 2009, Hayward *et al.* 2010, Dennis *et al.* 2011) we propose the following relationships between *C. forsteri* infection events, host response, and diagnosis as shown in Figure 5.7. As the egg stage of infection may be the main cause of pathogenesis in *C. forsteri* infection, detection of infection at the earliest stages would aid the development of treatment protocols. Currently, infection can only be described after flukes are present in the heart and/or after eggs are produced, which delays detection to 30-50 days post infection. While there were no adult flukes detected in wild *T. maccoyii* or *T.maccoyii* at the commencement of ranching in this study, a number of fish were found with eggs and an even greater number were positive for specific antibody activity. Serial non-destructive blood sampling followed by ELISA, may be used to determine the timing infection of immature flukes invading *T. maccoyii* host, however the method would need to be validated first. Detection of anti-fluke antibody could be useful in addition to parasite detection and in some cases can have 92-97% sensitivity (Tosswill & Ridley 1986), yet in *Schistosoma* infections in humans ELISA antibody detection cannot be reliably used until three months post exposure to the parasite (McLaren *et al.* 1978). Alternatives, such as detection of circulating antigen may be effective for diagnosis of infection at the earliest stages. In humans with Schistosomiasis, the expression profiles of the three life stages of the parasite, egg, cercariae and adult, were determined to be different at the mRNA and protein level, and cercarial antigen has been used to diagnose in humans as early as 21 days post infection (Zhou *et al.*

2010). In the identification of the intermediate host of *C. forsteri*, cercariae within the intermediate host was sequenced (Cribb *et al.* 2011). It may be possible to use this sequence to develop a PCR blood test to detect invading cercaria in *T. maccoyii*. In addition, it may also be possible to develop a PCR to detect the changes in antigen composition at the various stages of *C. forsteri* infection. Development of non-lethal and early detection of infection is needed to monitor the infection.

In conclusion, the timeline of *C. forsteri* infection in *T. maccoyii* was validated, with confirmation of infection occurring at the initiation of ranching and continuing throughout the entire ranching duration, fluke maturation occurring at approximately 28 d post infection with eggs originating from the heart and using blood flow to migrate to the gills for development. Immune response developed concurrently with *C. forsteri* infection, with the majority of physiological response coinciding with commencing egg production. Further research is needed to confirm the origin of *C. forsteri* antigen which is responsible for immune response development and how *T. maccoyii* immune response works against infection. To aide this research, further diagnostic methods for diagnosis of infection need to be developed.

Figure 5.7 Timeline of primary *C. forsteri* infection in *T. maccoyii*: fluke life stages associated physiological response and available diagnostic tests. Items in red have been added to our knowledge from this study. Those items in dotted boxes are hypothesized but have not been experimentally proven. The order of items within physiological response and diagnostic tests sections are from most effective/prevalent to less effect/rare. [[1]Aiken *et al.* 2009, [2]Colquittt 2001, [3]Colquittt *et al*. 2001, [4]Rough 2000, [5]Munday *et al.* 2003, [6]Hayward *et al.* 2010, [7]Dennis *et al.* 2011, [8]Aiken *et al.* 2006, [9]Colquittt 1999, [10]Shirakashi *et al.* 2012]


LIFESPAN: 30 TO 90 DAYS
3 to 9 days
21 to 47 days
3 to 66 days
Cercariae[1]
Migrating fluke[1] (*blood vessels*)
Juvenile fluke (*heart*)[1]
Adult fluke (*heart*)[1]
Eggs (*heart*)
Eggs (*gills*)
Focal granulomas (*heart*)
Antigen Recognition
Antigen Recognition
Antibody response (+ve)
(+ve)
ACH50 (-ve)
Lysozyme (+ve)
PHYSIOLOGICAL RESPONSE
Endocarditis (*heart*)[2]
thickening of myocardial fibres & decrease of lumen (*heart*)[2]
Granulomas , thrombi & oedemas (*gills*)[3]
Increased mucus on gills, respiratory distress and lethargy[4,5]
Slight mortality increase[4,5,6,7]
Parasitology: Heart flushes[8]
Histopathology (*heart* & *gills*)[2,3,9]
ELISA
DIAGNOSTIC TESTS
Wet Prep (*gills*)[10]
Gross Assessment (*gills*)[2]
Antigen detection
Antigen detection

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CHAPTER 6:

AN ANALYSIS OF TWO MANAGEMENT STRATEGIES FOR A BLOOD FLUKE *CARDICOLA FORSTERI* INFECTION IN RANCHED SOUTHERN BLUEFIN TUNA *THUNNUS MACCOYII*

Abstract

Two management strategies are described for the natural blood fluke, *Cardicola forsteri*, infection of Australian ranched Southern Bluefin Tuna (SBT), *Thunnus maccoyii*: (1) temporary offshore ranching and (2) chemotherapeutic treatment with Praziquantel. Both strategies were examined within a single cohort of SBT over a complete commercial ranching season. Effects on *C. forsteri* infection, SBT performance (i.e. survival and physical variables) and health (i.e. blood variables) were monitored pre- and post-treatment. Both management strategies successfully reduced infection and mortality. Evidence of reinfection and/or delayed infection was observed in both strategies, although at a significantly reduced rate than would be expected for naive fish. Several areas of further research to optimize and further understand the short-term and long-term effects of each management strategy are recommended.

6.1 Introduction

The blood fluke, *Cardicola forsteri*, has been a common infection in Australian ranched Southern Bluefin Tuna (SBT) since its discovery in 1997 (Cribb *et al*. 2000). The prevalence of infection increases from 5-10% at the initiation of ranching (Kirchhoff *et al*. 2011a) to 100% by two months of ranching, with an average of 27 adult flukes per SBT (Aiken *et al.* 2006). Previously, infection did not appear to result in clinical disease in SBT (Aiken *et al.* 2006, 2008; Colquittt *et al.* 2001), but recently an increase in infection intensities has coincided with elevated mortality (Dennis *et al.* 2011, Hayward *et al.* 2010). A mortality event has been described in Australian ranched SBT from approximately weeks 6 to 12 of ranching, resulting in an average cumulative mortality over the ranching year of 2-12% (Kirchhoff *et al.* 2011a, Dennis *et al.* 2011). Therefore, developing a commercial management plan to reduce *C. forsteri* infection became an industry priority.

C. forsteri (Trematoda Aporocotylidae) has a complex lifestyle, including a definitive host, bluefin tuna, and an intermediate host, a marine polychaete *Longicarpus modestus* (see Cribb *et al.* 2011). It is commonly believed that ranched SBT are continuously exposed to infectious cercariae throughout their ranching duration as both the intermediate and definitive host present within the current SBT farming zone (Aiken *et al.* 2009; Kirchhoff *et al.* (submitted)). All known cases of blood fluke related mortality are associated with the larval or egg stage of infection rather than adult (Bullard & Overstreet 2008). Therefore reducing contact between SBT and the infectious stage of *C. forsteri* and/or reducing infection within SBT prior to *C. forsteri* becoming mature may have an effect on reducing mortality as well as infection. In 2010, Kirchhoff *et al* (2011b) completed a trial to examine whether the industry's new move to offshore ranching of SBT resulted in the absence of *C. forsteri* infection. It was hypothesised that ranching in deeper water, further from shore than the current zone, may reduce or even eliminate contact between ranched SBT and the infectious cercariae. Yet maintenance of SBT pontoons so far from shore is expensive and risky, therefore an alternative strategy of leaving the pontoons temporarily offshore was developed. The first aim of this study was to determine if fish temporarily maintained offshore will maintain their *C. forsteri* free status, elevated physical condition earlier in the season, and reduced mortality after relocation near shore.

An experimental trial testing several anthelmintics against *C. forsteri* in SBT showed that Praziquantel was effective at eliminating adult infection *in vitro* and *in vivo* and reducing the quantity of eggs present in the heart *in vivo* (Hardy-Smith *et al.* 2012). The *in vivo* trial utilized stomach intubation, a method which is not commercially practicable. The ability to deliver Praziquantel through the normal diet of ranched SBT, baitfish, was therefore investigated. Injection of baitfish has been an effective method of delivering vitamins and immunostimulants to ranched SBT in the past (Kirchhoff *et al.* 2011c). In addition, the effect of Praziquantel treatment on reducing mortality remains unknown. The second aim of this project was to determine whether Praziquantel commercially delivered through injected baitfish reduces infection and mortalities.

6.2 Methods

6.2.1 Experimental fish and study design

Approximately 7500 wild SBT were captured by purse seine in the Great Australian Bight in January 2011. Following transport to the Tuna Offshore Farming Zone (TOFZ), SBT were transferred into four grow-out pontoons on 26 Feb 2011. Three of the grow-out pontoons were located offshore at 34º48.427'S 136º27.645'E and one was located near shore at 34º41.877'S 136º03.930'E. SBT were stocked at an initial density of approximately 3 kg m^{-3}. The near shore SBT were fed sardines at an average rate of 1.83 kg SBT^{-1} day^{-1} and offshore relocated SBT at an average rate of 1.72 kg SBT^{-1} day^{-1} for their entire ranching period. At 5 weeks (or 35 days) post-transfer, the near shore SBT were fed Praziquantel injected baitfish, at 75 mg kg^{-1} day^{-1} for two days (near shore treated SBT) . At 7 weeks (or 46 days) post-transfer, the offshore SBT were relocated from the offshore grow-out site to the near shore grow-out site (offshore relocated SBT). Results were analyzed on the basis of time post-transfer, time post-treatment and relative time post-relocation.

6.2.2 Sample Collection

6.2.2.1 Field Collection

Samples were collected opportunistically from 1.5 to 23.5 weeks post-transfer during commercial harvest; therefore sample size and timing of samples varied with commercial demand (Table 6.1). SBT were caught either by baited hook or by diver and chosen depending on the market demand. Offshore relocated SBT were sampled always from the same pontoon.

Once landed on the boat, SBT were immediately spiked in the head, brain removed using a 'Taniguchi tool' (core) and a wire placed down the spine to destroy the upper spinal nerves. Total time between capture and killing of each SBT was less than one minute. Fish were then bled by severing the pectoral artery behind the pectoral recess, and whole blood collected in a 9 ml heparinized Vacutainer ® tubes (BD, USA) and a 50 ml non-heparinized centrifuge tube, and placed on ice. Blood was collected within 1 minute of capture. The gills and viscera were then removed. A histological sample of gills was immediately collected from the second right gill arch and from the heart ventricle, atrium, and bulbous arteriosus and placed in 10% neutral buffered formalin. The heart was then placed in a waterproof tub and stored on ice. The SBT carcass was then placed in ice slurry. Fork length (cm) and gilled and gutted weight (kg) were recorded for all SBT at the processing plant within 24 hours of capture. Condition index was calculated for each SBT using the formula: [gilled and gutted weight (kg) /0.87] / [length (m) ^3] (Hayward *et al*. 2010), which converts gilled and gutted weight to whole weight for SBT. Optimal harvest condition occurs at condition index values greater than 24. Mortalities were assessed daily by commercial divers.

Table 6.1 Sampling time points and sample sizes (n) for each of the two treatments, nearshore and offshore. n.a. denotes samples which were not available.

	Nearshore			Offshore		
	n	**description**	**Capture method**	**n**	**description**	**Capture method**
Week 1.5	10	Baseline	hook and line	10	Baseline	hook and line
Week 5	10	Pre-treatment	hook and line	n.a.		
Week 7	n.a.			7	1 day post relocation	hook and line
Week 9.5	5	4.5 weeks post treatment	hook and line	20	2 weeks post relocation	diver
Week 10.5	n.a.			20	3.5 weeks post relocation	diver
Week 11.5	20	6.5 weeks post treatment	diver	8	5 weeks post relocation	hook and line
Week 13	20	8 weeks post treatment	diver	n.a.		
Week 13.5	20	8.5 weeks post treatment	diver	9	6.5 weeks post relocation	hook and line
Week 20.5	n.a.			20	Harvest, 13.5 weeks post relocation	diver
Week 23.5	20	Harvest, 18.5 weeks post treatment	diver	n.a.		

6.2.2.2 Laboratory Processing

The heparinized vial of whole blood was used for whole blood and plasma aliquots. Three 500 µl aliquots of whole blood were transferred into 1.5 ml plastic tubes and frozen at -20°C. The remaining blood was centrifuged at 3000xg at 4°C for 10 min. Blood plasma was aliquoted into four 1.5 ml plastic tubes, and frozen at -20°C. The non-heparinized vial of whole blood was used for serum collection. Vials were

stored upright at 4°C for 24 h and serum aliquoted into three-1.5 ml tubes. If the serum could not be removed without disturbing the blood clot, the samples were centrifuged at 1000xg at 4°C for 5 min. Serum samples were frozen at -20°C. Hearts of ranched SBT were dissected 2–4 h after removal from the carcass and flushed with physiological saline to dislodge any adult *Cardicola forsteri* (see Aiken *et al*, 2006). Flushes were then poured into Petri dishes and examined for the presence of adults using a dissecting microscope.

6.2.3 Laboratory Analysis

6.2.3.1 Hematology

Hemoglobin concentrations were determined from whole blood aliquots using the cyanometahemoglobin assay based on Brown (1984) modified by Kirchhoff *et al*, (2011c). Whole blood plasma glucose and lactate were measured using Accu-Chek ® Advantage II and Accutrend ® Plus by Cobas, respectively. The pH of blood plasma samples was measured using a Minilab Isfet pH meter Model IQ125 (IQ Scientific, USA). Blood plasma osmolality was determined using a Vapro© Model 5520 vapour pressure osmometer (Wescor Inc., Logan, Utah, USA).

6.2.3.2 Humoral Immune Response

Blood serum was analyzed in triplicate for lysozyme activity and alternative complement activity. Lysozyme activity was measured using a method based on Carrington and Secombes (2007) modified by Kirchhoff *et al*. (2011c). Blood serum alternative complement activity was measured using a modified Yano (1992) method as described by Kirchhoff *et al*. (2011c). Antibody presence specific to *Cardicola forsteri* was measured using an ELISA (Aiken *et al.*2008; Kirchhoff *et al.* (submitted)). Antigen and primary antibody concentrations followed the method by Aiken *et al.* (2008). Coating antigen and sample concentrations were optimized using a checkerboard technique before running ELISA. *C. forsteri* antigen was prepared using the methods outlined by Aiken *et al*. (2008). Iwaki ELISA 96-well flat bottom plates were coated with 2.5 µg well^{-1} antigen in coating carbonate buffer, covered with parafilm, and incubated overnight at 4ºC. The plates were then washed 3x with low salt wash buffer (2.42g Trisma base, 22.22g NaCl, and 0.5ml Tween in 1l dH_2O at pH 7.3) and blocked with 250µl well^{-1} 3%w/v dried milk in dH_2O for 2h at 22ºC. The plates were then washed 3x with low salt wash buffer, 100 µl well^{-1} sample and control serums 1:100 in PBS were added in duplicate, and incubated for 3h at 22ºC. Triplicate seropositive and seronegative controls were run on each plate at 1:2 to 1:128 four-fold and eight-fold serial dilutions, respectively. Plates were washed 5x with high salt wash buffer (2.42g Trisma base, 29.2g NaCl, and 1ml Tween in 1l dH_2O at pH 7.7), incubated for 5min on the last wash, 100 µl well^{-1}

Anti-Bluefin Tuna (*Thunnus thynnus*) monoclonal antibody (Product No. F19, Aquatic Diagnostics Ltd) 1:50 in antibody buffer (1g BSA in 100ml PBS) was added, and incubated for 1h at 22ºC. Plates were washed 5x with high salt wash buffer, incubating for 5min on the last wash, and then plates were developed. Bound antibody was visualized by adding 100 µL of chromogen substrate, 3,3´,5,5´-tetramethylbenzidine (TMB One Solution, Promega, Fitchburg, WI, USA), incubated for 10 min at RT and stopped with 50 µL of 2 M H_2SO_4 solution in dH_2O. Absorbance was read at 450 nm in a Rainbow Thermo plate reader (Tecan Group Ltd., Männedorf, Switzerland). Sample antibody concentration (µl serum ml^{-1}) was calculated by comparison of absorbance value to the 4-variable logistic curve generated using seropositive control absorbance values from the same plate. An individual was defined as positive if anti*Cardicola* antibody activity when titre > 1 unit μl^{-1}.

6.2.4 Statistical Analysis

At each sampling point *C. forsteri* infections were characterized by prevalence (the number of host infections as a proportion of the population at risk) and mean intensity (the average number of parasites in infected hosts) (Bush *et al*, 1997). Presence of Anti*Cardicola* antibody activity was determined by prevalence (the number of hosts positive for immunoglobulin activity as a proportion of the total population). Sterne's exact 95% confidence intervals were calculated for prevalence, and 95% bootstrap confidence intervals (with 2000 replications) were calculated for mean intensity, using the software 'Quantitative Parasitology 3.0', supplied by Reiczigel & Rózsa (2005). The prevalence and mean intensity were compared between management strategies and ranching durations in a pair-wise fashion. When comparing management strategies with time adjusted data, the timing of ranching duration was readjusted so that the first day after Praziquantel treatment or relocation corresponded to day one of ranching. Given the high total number of pairwise comparisons, $\alpha = 0.01$ was regarded as significant for these statistics.

All blood and physical condition results were interpreted using the R 2.12.2 statistical package (©2011, The R Foundation for Statistical Computing). Survival was assessed using log-rank test for equality of the two Kaplan-Meier survival curves, one for each management strategy. Survival data were analysed accounting for all fresh harvests, which occurred periodically from each management strategy throughout

the ranching duration. To describe the change in physical and blood variables over the ranching duration, a one-way ANOVA with a Tukey's HSD post-hoc test was completed for each management strategy. The assumptions of homogeneity of variances and normalcy were checked by the Residuals vs. Fitted and Q-Q plots. Any outliers were checked for data collection accuracy, i.e. laboratory analysis repeatability, and if values fell within a known physiological range for SBT, prior to deciding to remove a data point. Anti*Cardicola* antibody activity was $\log_{10}+1$ and rank transformed prior to statistical analysis due to their failure to conform to normality and homogeneity of variances. A significance level of $\alpha=0.05$ was used for all analysis. Significance was described when a blood variable fell outside the normal physiological range (Table 6.2).

Table 6.2 The accepted normal physiological range for various blood variables in Australian ranched SBT.

Blood variable	Physiological Range	Reference
Hb (g dl^{-1})	15-25	Kirchhoff *et al*., 2011a, 2011b, 2011c
Plasma pH	7.6-8.25	Thomas *et al*., 2003 Kirchhoff *et al*., 2011a, 2011b, 2011c
Osmolality (mmol kg^{-1})	380-480	Kirchhoff *et al*., 2011a, 2011b, 2011c
Glucose (mmol l^{-1})	3-8	Kirchhoff *et al*., 2011a, 2011b, 2011c
Lactate (mmol l^{-1})	4-11	Thomas *et al*., 2003 Kirchhoff *et al*., 2011a, 2011b
Lysozyme (µg ml^{-1})	25-150	Kirchhoff *et al*., 2011a, 2011b, 2011c
ACH50 (units ml^{-1})	100-170	Kirchhoff *et al*., 2011a, 2011b, 2011c

6.3. Results

6.3.2 Offshore relocated SBT

Cumulative mortality during the 20.5 weeks of ranching was 1.138%. Mortality from transfer until relocation of the pontoon at 7 weeks of ranching was 0.1%, equivalent to 2 SBT (Figure 6.1). No additional mortality occurred until week 19 of ranching, corresponding to 13 weeks post relocation. Most mortalities occurred from week 19 to 20 (Figure 6.1). It is important to note that the offshore relocated pontoon was reduced to 300 fish directly after relocation, and therefore the cumulative mortality from week 19-20 was equivalent to 4 SBT.

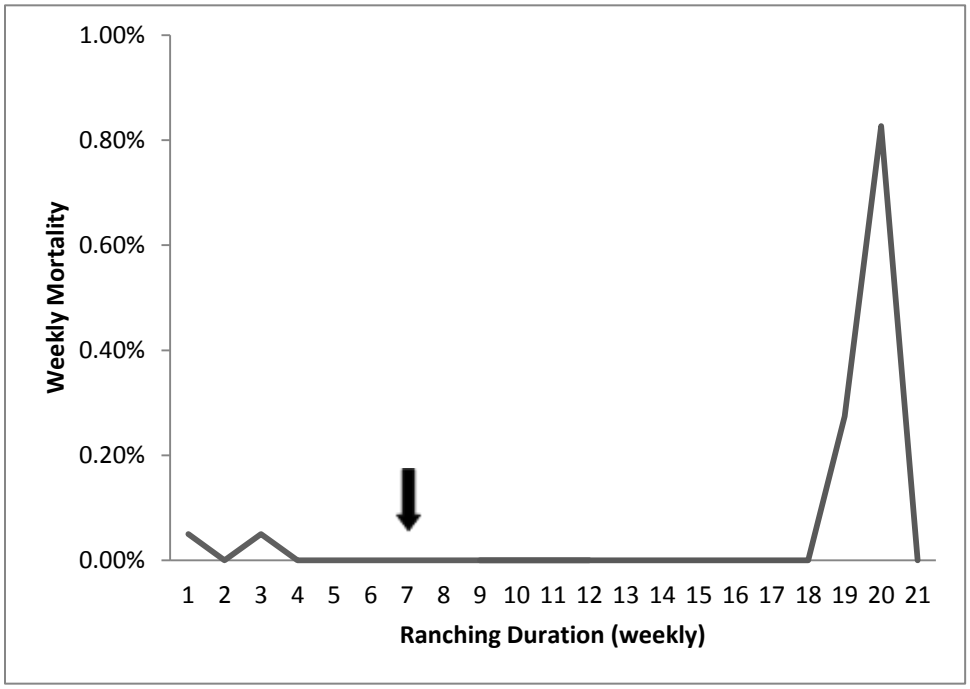

Figure 6.1 Offshore relocated SBT weekly mortality calculated as percentage of the number of fish remaining at the start of each week. Arrow denotes timing of relocation, which also corresponds to timing of pontoon population reduction to 300 SBT.

No *C. forsteri* were found prior to relocation. The prevalence and mean intensity of *C. forsteri* increased over the ranching duration (Table 6.3). Adult *C. forsteri* were present from week 11 of ranching, or 4 weeks post relocation, and increased in prevalence to 66.7% from week 13 of ranching, or 6 weeks post relocation. Mean intensity increased to 5.95 by harvest, at 20.5 weeks of ranching.

Weight, length and condition index of sampled SBT increased over the ranching duration (Table 6.4). Weight increased by 15.9 kg (gg) by week 10.5 and 22.8 kg (gg) by week 20.5 of ranching ($F=10.692$, $df=6,86$, $p<0.001$). There was a significant increase in length of SBT ($F=5.0691$, $df=6,86$, $p<0.001$). A harvest equivalent condition was achieved by week 10 of ranching ($F=42.78$, $df=6,86$, $p<0.001$).

Table 6.3 Summary for prevalence (95% confidence interval) and mean intensity (95% confidence interval) for *Cardicola forsteri* infection in offshore relocated SBT. Week 7 corresponds with 1 day post relocation. Different letters denote statistical difference within prevalence or intensity, respectively, at α=0.01. n.a. denotes results which were not available to be calculated.

	Prevalence (%)	Mean intensity
Week 1.5	0.0 (0.0-35.0)[a]	0.00 (n.a.)
Week 7	0.0 (0.0-37.7)[a]	0.00 (n.a.)
Week 9.5	0.0 (0.0-16.7)[a]	0.00 (n.a.)
Week 10.5	0.0 (0.0-17.6)[a]	0.00 (n.a.)
Week 11.5	12.5 (0.6-50.0)[ab]	5.00 (n.a.)
Week 13.5	66.7 (32.3-90.2)[bc]	1.50 (1.00-2.17)[a]
Week 20.5	100.0 (83.3-100.0)[c]	5.95 (4.75-7.40)[b]

Blood variables changed significantly, specifically between week 9 and week 13.5 of ranching, corresponding to 2 to 6.5 weeks post relocation (Table 6.4). Hemaoglobin was elevated 20% above normal between weeks 9 and 13.5 of ranching and 35% above normal at harvest, 20.5 weeks of ranching ($F=22.939$, $df=6,86$, $p<0.001$). Plasma pH was low at week 13.5, yet within physiological range at all other sample time points ($F=6.4645$, $df=6,87$, $p<0.001$). Plasma osmolality was elevated at week 9, 10.5 and 20, returning to normal physiological range in weeks 12 and 13.5 ($F=22.854$, $df=6,87$, $p<0.001$). Plasma glucose was 60% above normal at week 9 and 10.5 ($F=20.172$, $df=6,87$, $p<0.001$). Plasma lactate level increased at week 9 ($F=9.3268$, $df=6,87$, $p<0.001$). Lysozyme concentration was elevated ~1.5x from week 9 through 12 and in week 20.5, returning to physiological normal range in week 13.5 ($F=3.839$, $df=6,87$, $p=0.002$). Alternative complement activity was reduced by 25% at week 9 and by 50% at week 10.5, returning to normal physiological range by week 11.5 ($F=11.857$, $df=6,86$, $p<0.001$). Anti*Cardicola* antibody level was low in SBT sampled at week 9 but then recovered by harvest ($F=3.2899$, $df=6,87$, $p=0.006$).There was no change in Anti*Cardicola* prevalence ($p>0.01$).

Table 6.4 Physical and blood variables in offshore relocated SBT from 1.5 to 20.5 weeks of ranching. Relocation from an offshore to near shore ranching site occurred at 7 weeks of ranching, denoted by the double line. Different letters denotes statistical differences within a variable over ranching duration.

OFFSHORE RELOCATED							
	Ranching duration (week post transfer)						
	1.5	**7**	**9.5**	**10.5**	**11.5**	**13.5**	**20.5**
Weight (gg)	19.4±1.4^{a}	27.4±2.0^{ab}	35.3±1.5^{bc}	34.5±1.9^{b}	35.8±2.5^{bc}	38.3±3.1^{bc}	42.2±3.1^{c}
Length (cm)	107.9±2.8^{a}	111.9±2.8^{ab}	120.3±2.0^{bc}	116.5±2.1^{abc}	119.8±2.7^{abc}	121.8±3.5^{bc}	124.4±1.8^{c}
Condition index	17.50±0.19^{a}	22.23±0.49^{b}	23.59±1.18^{b}	24.69±0.30^{c}	23.76±0.46^{bc}	23.97±0.46^{bc}	24.89±0.29^{c}
Hb (g dl^{-1})	18.88±0.36^{a}	22.48±0.84^{b}	25.98±0.42^{c}	24.95±0.86^{bc}	26.60±0.66^{cd}	24.46±0.57^{bc}	29.18±0.47^{d}
Plasma pH	7.69±0.04^{abc}	7.83±0.06^{bc}	7.68±0.06^{ab}	7.85±0.04^{bc}	7.76±0.04^{bc}	7.46±0.08^{a}	7.88±0.04^{c}
Osmolality (mmol kg^{-1})	405.3±2.4^{a}	423.3±3.3^{a}	495.6±7.0^{b}	492.1±7.9^{b}	423.1±11.8^{a}	411.3±2.5^{a}	468.9±7.9^{b}
Glucose (mmol l^{-1})	7.12±0.11^{a}	7.16±0.23^{a}	10.27±0.30^{b}	11.45±0.62^{b}	7.03±0.13^{a}	7.76±0.19^{a}	7.95±0.17^{a}
Lactate (mmol l^{-1})	8.89±0.25^{a}	8.43±0.38^{a}	10.72±0.24^{b}	9.23±0.36^{a}	7.75±0.31^{a}	8.69±0.39^{a}	8.42±0.27^{a}
Lysozyme (µg ml^{-1})	90.38±12.22^{a}	143.32±17.78^{a}	190.40±15.58^{b}	190.01±16.58^{b}	226.31±34.17^{b}	153.99±16.26^{a}	211.61±23.51^{b}
ACH50 (units ml^{-1})	197.34±16.79^{ab}	131.93±15.22^{abcd}	87.97±11.21^{cd}	41.28±10.01^{d}	151.05±23.69^{abc}	114.48±10.17^{bcd}	214.73±28.22^{a}
Anti*Cardicola* (µl ml^{-1})	20.748±6.009^{ab}	46.285±17.896^{ab}	8.898±3.745^{b}	11.957±5.284^{ab}	32.914±13.667^{ab}	9.163±4.460^{ab}	62.929±21.833^{a}
Anti*Cardicola* (%)	60 (29-85)	71 (34-95)	30 (14-53)	50 (14-53)	88 (56-99)	88 (56-99)	80 (58-93)

6.3.2 Near shore treated SBT

Cumulative mortality during the 23.5 weeks of ranching was 0.766%. Mortality from transfer until Praziquantel treatment at 5 weeks of ranching was 0.31%, equivalent to 10 SBT. Mortality remained at a low level, ~0.01% per week until week 10 of ranching or 5 weeks post treatment (Figure 6.2). Cumulative mortality post treatment was 0.456%, equivalent to 12 SBT.

No *C. forsteri* were found within near shore SBT prior to treatment. The prevalence of *C. forsteri* increased over the ranching duration (Table 6.5). *C. forsteri* were present from week 9.5 of ranching, corresponding to 4.5 weeks post treatment. Prevalence remained at <20% from weeks 9.5 to 13.5 of ranching, corresponding to weeks 4.5 to 8.5 post treatment. Prevalence increased to 70% and mean intensity increased to 5.57 by harvest, at 23.5 weeks of ranching or 18.5 weeks post treatment.

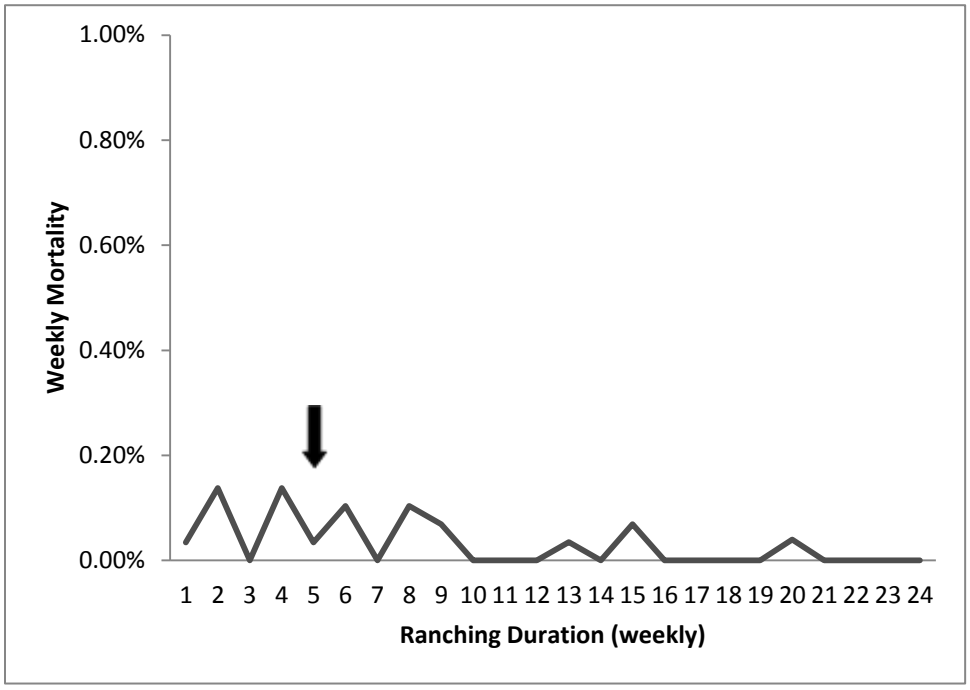

Figure 6.2 Near shore Praziquantel treated SBT weekly mortality calculated as percentage of the number of fish remaining at the start of each week. Arrow denotes time of Praziquantel treatment.

Weight, length and condition index of sampled SBT increased over the ranching duration (Table 6.6). Average weight of the sampled SBT increased 13.3 kg (gg) by week 11.5 and 30.8 kg (gg) by week 23.5 of ranching ($F=20.668$, $df=6,95$, $p<0.001$). Average length of sampled SBT increased by week 13 of ranching ($F=10.97$, $df=6,94$, $p<0.001$). A harvest equivalent condition was achieved between week 13.5 and week 23.5 of ranching ($F=16.449$, $df=6, 94$, $p<0.001$). No significant changes in physical parameters were noted prior to treatment.

Table 6.5 Summary for prevalence (95% confidence interval), mean abundance (95% confidence interval) and mean intensity (95% confidence interval) for *Cardicola forsteri* infection in near shore treated SBT. Week 5 corresponds with pre-treatment and week 9.5 with 4.5 weeks post treatment. Different letters denote statistical difference within prevalence or intensity, respectively, at α=0.01. NA denotes the result was not computable.

	Prevalence (%)	Mean intensity
Week 1.5	0.0 (0.0-35.0)[a]	0.00 (NA)
Week 5	0.0 (0.0-35.0)[a]	0.00 (NA)
Week 9.5	20.0 (1.0-65.7)[ab]	1.00 (NA)
Week 11.5	15.0 (4.2-37.2)[a]	1.33 (1.00-1.67)
Week 13	10.0 (1.8-32.0)[a]	1.50 (1.00-1.50)
Week 13.5	15.0 (4.2-37.2)[a]	1.0 (NA)
Week 23.5	70.0 (47.5- 86.0)[b]	5.57 (3.21-9.50)

Table 6.6 Physical and blood variables in near shore treated SBT from 1.5 to 23.5 weeks of ranching. Treatment with Praziquantel occurred at 5 weeks of ranching, denoted by the double line. Different letters denotes statistical differences within a variable over ranching duration.

NEAR SHORE TREATED							
	Ranching duration (week post transfer)						
	1.5	**5**	**9.5**	**11.5**	**13**	**13.5**	**23.5**
Weight (gg)	21.8±1.4^{a}	27.6±1.6^{ab}	24.8±0.8^{ab}	35.1±1.6^{b}	37.3±1.8^{b}	36.5±2.2^{b}	52.9±2.6^{c}
Length (cm)	108.9±2.0^{a}	115.7±3.0^{abc}	106.8±2.5^{ab}	119.4±1.9^{abc}	121.9±1.8^{c}	120.3±2.7^{bc}	134.3±2.5^{c}
Condition index	19.12±0.47^{a}	20.46±0.58^{a}	23.14±0.33^{b}	23.53±0.32^{b}	23.35±0.27^{b}	23.69±0.33^{b}	24.99±0.56^{b}
Hb (g dl^{-1})	21.48±1.23^{a}	22.79±1.04^{a}	22.88±1.02^{ab}	27.26±0.73^{bc}	27.15±0.44^{bc}	27.70±1.12^{bc}	26.29±0.69^{c}
Plasma pH	7.65±0.02^{b}	7.72±0.02^{bc}	7.80±0.09^{bc}	7.95±0.05^{c}	7.38±0.05^{a}	7.72±0.04^{b}	7.79±0.05^{bc}
Osmolality (mmol kg^{-1})	406.3±3.9^{a}	410.5±3.1^{a}	421.4±7.0^{ab}	508.2±7.7^{d}	466.3±6.1^{bc}	457.2±7.5^{bc}	485.5±10.7^{cd}
Glucose (mmol l^{-1})	6.93±0.09^{a}	6.85±0.14^{a}	6.88±0.14^{a}	8.97±0.28^{b}	9.00±0.24^{b}	8.79±0.19^{b}	8.50±0.18^{b}
Lactate (mmol l^{-1})	9.07±0.20^{abc}	9.54±0.31^{bc}	7.98±0.33^{ab}	8.14±0.29^{a}	9.91±0.29^{c}	9.50±0.23^{bc}	9.06±0.28^{abc}
Lysozyme (µg ml^{-1})	111.09±13.91	141.85±12.31	126.09±27.07	172.61±15.77	161.13±22.10	156.97±11.74	155.38±19.08
ACH50 (units ml^{-1})	194.35±6.85^{ab}	156.95±9.07^{ab}	116.97±14.30^{ab}	122.57±46.91^{ab}	88.49±10.46^{b}	106.00±24.76^{b}	251.95±54.54^{a}
Anti*Cardicola* (µl ml^{-1})	18.694±4.845^{ab}	8.110±5.541^{c}	0.000±0.000^{c}	25.050±3.926^{b}	22.568±13.878^{c}	16.147±8.587^{c}	34.812±7.057^{a}
AntiCardicola (%)	70 (38-91)^{ab}	20 (4-55)^{b}	0 (0-50)^{b}	95 (76-99)^{a}	55 (32-76)^{b}	45 (24-68)^{b}	95 (76-99)^{a}

Blood variables changed significantly, specifically between week 11.5 and week 13 of ranching, corresponding to 6.5 to 8 weeks post treatment (Table 6.6). Hemoglobin was elevated 20% above physiological norm from week 11.5 to harvest ($F=7.5456$, df=6,98, $p<0.001$). Plasma pH was low at week 13, yet was within physiological range at all other sample times ($F=14.557$, df=6,98, $p<0.001$). Plasma osmolality was elevated at week 11.5, declining slightly yet remaining elevated until harvest ($F=18.910$, df=6,98, $p<0.001$). Plasma glucose was elevated 30% above physiological norm from week 11.5 to harvest ($F=14.792$, df=6,98, $p<0.001$). Although plasma lactate changed over ranching duration ($F=5.4253$, df=6,98, $p<0.001$), values remained within the normal physiological range. Alternative complement activity was reduced by 25% from weeks 13 to 13.5, yet returned to a level slightly elevated from physiological norm by harvest ($F=2.6091$, df=6,97, $p=0.022$). Anti*Cardicola* antibody prevalence and level changed cyclically over the ranching duration ($F=8.3147$, df=6, 98, $p<0.001$): decreasing to low prevalence and levels from 5 to 9.5 weeks, increasing at 11.5 weeks, decreasing again from 13 to 13.5 weeks, and then increasing at harvest. Lysozyme concentration did not change. No blood variable changes were noted prior to Praziquantel treatment.

6.3.3 Comparison between near shore treated and offshore relocated SBT

There was no difference in survival between near shore treated and offshore relocated SBT ($X^2=0$, df=1, $p=0.849$). The offshore relocated SBT had a 4x higher prevalence of *C. forsteri* from week 13 of ranching compared to near shore treated SBT. When dates were adjusted so that Praziquantel treatment and relocation occurred at the same time, then offshore relocated SBT maintained a higher prevalence from 7 to 14 weeks post treatment/relocation. The timing of infection was similar in both management strategies, with evidence of infection at week 4 post treatment or post relocation. No differences in mean intensity were found between the two management strategies as infection loads were low across both strategies.

6.4 Discussion

Two successful management strategies for natural *C. forsteri* infection in SBT have been described (1) temporary offshore holding and (2) Praziquantel treatment. Both strategies successfully reduced infection and mortality within ranched SBT in comparison to those experiences in the last few years (Hayward *et al.* 2010, Dennis et al 2011, Kirchhoff *et al.* 2011a, 2011b, 2011c). At the same time both management strategies showed evidence of limited effectiveness in a long term, with *C. forsteri* reinfection and/or delayed infection occurring in both strategies.

6.4.1 Temporary Offshore holding

Ranching SBT offshore for 7 weeks successfully delayed *C. forsteri* infection, reduced mortality, and maintained enhanced physical condition earlier in the ranching season. This confirms results from the previous ranching season showing that there is no *C. forsteri* infection in offshore held SBT, possibly due to the lack of intermediate host or greater depth increasing the physical separation between the final and intermediate host if present (Kirchhoff *et al.* 2011b). There were no indications that relocation caused additional stress, as determined by maintained physical condition and lack of significant changes in blood variables immediately following relocation. If ranching duration was adjusted for the timing of relocation, infection occurred on the same timescale as newly arrived, previously uninfected SBT (Aiken *et al.* 2006, Kirchhoff *et al.* 2011a, 2012).

Temporary offshore ranching may provide a 'buffering' capacity to SBT, protecting them against infection while improving their condition and aiding their ability to resist and respond to *C. forsteri* infection. The blood variables did indicate the possibility of a stress or immune compromise from week 9 through to 10.5. At this time, glucose, lactate, and osmolality were found to be elevated as well as increased lysozyme and decreased alternative complement activity. These changes are not similar to the expected blood variables changes during the 6-12 week mortality event in Australian ranched SBT, when a reduced concentration of hemoglobin, lysozyme activity and alternative complement activity were observed (Kirchhoff *et al.* 2011a).

6.4.2 Praziquantel treatment

Praziquantel treatment successfully delayed infection. Praziquantel may have effectively 'reset the clock', with infection appearing at the same time post-treatment as post transfer for SBT arriving to the ranching zone (Kirchhoff *et al.* 2011a, Aiken *et al.* 2006), at approximately 4 weeks post treatment. However, the intensity of infection was significantly lower than that expected for naive fish (Aiken et al 2006), therefore treatment with Praziquantel may promote long-term protection. This is the first time successful administration of Praziquantel through injected baitfish has been reported in scientific literature.

The minimum effective Praziquantel dose against *C. forsteri* is currently unknown. Prior research determined significant effects against *C. forsteri* at Praziquantel doses of 75 mg kg SBT^{-1} utilising stomach intubation *in vivo* and at concentrations greater than 0.0625 µg ml^{-1} *in vitro* (Hardy-Smith *et al* 2012). Adult *Schistosoma mansoni* flukes treated with Praziquantel *in vitro* were found to recover if transferred into drug-free media 1 to 4 hours post treatment (Xiao et al 2009). Praziquantel is known to clear considerably within 48 h from treated rockfish, *Sebastes schlegeli* (Kim et al 2001) and within 24 h from kingfish, *Seriola lalandi* (Tubbs and Tingle 2006) after treatment. Further studies are needed to optimize oral treatment delivery, dose, duration of treatment, and timing of treatment in ranching.

It is possible that the individual variability in antibody levels may be partially responsible for the infection post-treatment. In the killing of schistosomes, the efficacy of Praziquantel depends, at least in part, on the integrity of the host's immune system. In mice with a depleted β-lymphocyte population, the efficacy of Praziquantel was reduced from 81% to 17.5% (Brindley & Sher 1987). Therefore, it is hypothesised that the mode of action of Praziquantel requires a synergy with specific antibody response directly against the parasite antigens (Mutapi *et al*. 2005, Josteph *et al.* 2004, Brindley & Shear 1987). There was a large drop in specific antibody presence and level just prior to and 4 weeks after treatment. Further research would be required to determine if the drop in circulating *C. forsteri* specific antibodies immediately prior to and post treatment was due to a drop in production of antibodies by the host, an immune evasion/suppression strategy employed by the parasite, or due to increased attachment of antibodies to the flukes. The rapid binding of antibodies is also believed to explain low antibody levels in common carp, *Cyprinus carpio,* infected with the blood fluke *S. inermis* (Roberts *et al.* 2005*).* Yet, it appears the

treatment did not hinder the development of antibody activity when compared to the offshore relocated SBT in this study or to previous studies (Kirchhoff *et al.* 2012, Aiken *et al*. 2008). Praziquantel treatment is known to cause an increase in the number and array of exposed antigens on a schistosome tegument (Mutapi *et al.* 2005), aiding development of specific antibody response both quantitatively and qualitatively (Mutapi *et al.* 2005, Joseph *et al.* 2004). There is a lack of information about the effects of Praziquantel on *C. forsteri* antigens and antibody development.

C. forsteri infection was observed within this study at 4.5 weeks post treatment, indicating a potential need to repeat Praziquantel treatment during the ranching period. Although Praziquantel is effective against adult flukes, it has been show to be ineffective against developing schistosomes (Gray *et al*. 2010) and miracidia (Shirakashi *et al.* 2012). Reinfection was also noted in juvenile Pacific bluefin tuna, *Thunnus orientalis*, infected with *C. orientalis* and *C. opisthorchis*, with infection reappearing 3 to 5 weeks post Praziquantel treatment (in feed, surface coated). Miracidia of both *C. orientalis* and *C. opisthorchis* were found to be unaffected by Praziquantel treatment (Shirakashi *et al.* 2012). The *in vitro* trials completed by Hardy-Smith *et al*. (2012), described effectiveness of Praziquantel against only adult flukes due to the lack of availability of other life stages at the time of testing. Further research is needed to describe effectiveness against eggs, miracidia, and, if isolated, cercariae as well as the need for additional treatments.

6.4.3 Both management strategies

The delayed *C. forsteri* infection of the offshore relocated SBT or the infection at week 4.5 post treatment of the near shore treated SBT did not coincide with a mortality event, as was previously observed with *C. forsteri* infection (Hayward *et al.* 2010, Dennis *et al.* 2011). It is possible that the timing of *C. forsteri* infection and the mortality event may not be directly associated as previously described. *C. forsteri* infection/reinfection was detected at week 4.5 post treatment for the near shore treated SBT and 5 weeks post-relocation for the offshore relocated SBT, with the timing of the mortality event expected to occur between week 6 and 12 . No mortality was evident in either management strategy at this time, although infection prevalence and intensity was similar to 2009 and 2010 (Kirchhoff *et al* 2011a, 2011b, 2011c). It is possible that the 6-12 week mortality event is due to a combination of *C. forsteri* infection and another

presently unknown factor. If that was the case, by uncoupling the timing of these two events, we are preventing the mortality event from occurring altogether. Declining water temperatures may delay mortality even within the warm-blooded SBT so it occurs later than 6-12 weeks, and therefore the mortality could be masked by an early harvest as the offshore relocated SBT were harvested 13.5 weeks post relocation. An elevation in alternative complement activity was observed in both management strategies at the harvest sampling. Kirchhoff *et al.* (2011a) observed a rapid elevation in alternative complement activity just prior to the 6-12 week mortality in Australian ranched SBT. In addition, the offshore relocated SBT had some mortality just prior to harvest. Even if the mortality in offshore relocated SBT occurred sometime after 20 to 23 weeks of ranching, it may only be relevant if SBT are ranched for a longer duration.

There were several changes in blood variables which were shared between both management strategies: a decrease in plasma pH, an elevation in osmolality, and an increase in hemoglobin concentrations. A low plasma pH at week 13 was also observed in both management strategies. The observed blood acidosis may be associated with a large storm event which occurred from 22-24 May 2011. The 48-hour storm had wind gusts of 81 km h^{-1} and was a once in a decade event for the region (Australian Government Bureau of Meteorology, http://www.bom.bom.gov.au). The 13 week samples were taken 5 days in the near shore treated and 7 days offshore relocated SBT after the beginning of the storm event.

An elevation in osmolality was previously described in Australian ranched SBT (Kirchhoff *et al.* 2011c). This increase in osmolality is a sign of an osmoregulatory stress. Due to the timing of elevated osmolality and the stage of *C. forsteri* infection, it is unlikely to be associated with hatching *C. forsteri* eggs and their disruption of the gill epithelium. There were also no observed negative long-term effects of elevated osmolality on SBT physiology in this study; therefore this observation is most likely not biologically or commercially significant.

SBT from both management strategies had elevated hemoglobin concentrations at the later stages of ranching. Previously it was observed hemoglobin concentration was negatively related to *C. forsteri* infection intensity (Hayward *et al.* 2010), therefore an absence of infection may allow for the observation of the natural, previously undescribed, hemoglobin concentration in ranched SBT. Yet, in this study, it is

unlikely an absence of infection was the sole cause of the elevated hemaoglobin concentration, since the concentration remained high until harvest even though SBT became increasingly infected. The timing of this elevation was associated with increased condition factor. In birds, concentration of hemoglobin in the blood is strongly linked to food supply (Kilgas *et al.* 2006, Bańbura *et al.* 2007, 2008, Kalinski *et al.* 2009) This relationship is not as well described in fish, yet some observations in catfish, *Silurus triosteous*, show a positive relationship between fish weight and hemoglobin concentration (Al-Abood, 1989). Hemoglobin has been described as an indicator of long-term physiological condition (O'Brien *et al*. 2001, Nadolski *et al.* 2006, Slomczynski *et al.* 2006, Bańbura *et al.* 2007, 2008), therefore it is reasonable to assume elevated hemaoglobin concentration is associated with increased physical condition in SBT. Another possibility is that dehydration suggested by the increased osmolality caused a concentration in red blood cells, therefore elevated hemaoglobin concentration.

In conclusion, both management strategies successfully reduced infection and mortality in ranched SBT. While there was some evidence of reinfection and/or delayed infection in both strategies, this was at a reduced rate than would be expected for naive fish. Therefore, these management strategies may also promote long-term protection.

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CHAPTER 7:

GENERAL DISCUSSION

This research utilized current and newly acquired knowledge about southern bluefin tuna (SBT) health to make educated manipulations of the ranching environment and/or husbandry practices within the Australian southern bluefin tuna ranching industry. This project required refining the methods for blood, tissue, and parasitology analysis and resulted in a definition for 'normal' and 'abnormal' within each of the 15 various parameters described within the context of various environmental conditions. This thesis described changes in weight, length, condition index, blood variables, and immune response in relation to vitamin and immunostimulant supplementation (Chapter 2, (Note: Ridley Corporation Ltd., who supplied the immunostimulants used in the experiment, in consultation with co-author D'Antignana chose the concentrations of vitamins and immunostimulants used in Chapter 2 in commercial confidence)), an acute mortality event and ranching (Chapter 3), and to moving cages further offshore (Chapter 4). In addition, the natural infection timeline of blood fluke *Cardicola forsteri* was further described to include relationships between infection events, physiological SBT health and survival (Chapter 5) and several management strategies were proposed to prevent infection (Chapter 6).

Understanding the health of wild SBT and how it may affect their behavior has implications for international stock management and conservation efforts. Although there have been numerous studies concerning the schooling behavior (Hanrahan & Juanes 2001, Beare *et al.* 2003, Kirby *et al.* 2003, Newlands *et al.* 2006, Torisawa *et al.* 2007, Dell & Hobday 2008, Newlands & Porcelli 2008, Sabate *et al.* 2010, Fukuda *et al.* 2011) and health of tuna (Mladieno *et al.* 2008, Munday *et al.* 2003, Nowak *et al.* 2006), none have attempted to explain the effect of health on behavior. This lack of knowledge is due, in part, to the limitation of sampling and analytical techniques to define the health status of individual fish. Such hurdles have been overcome by this thesis, which provided key information on the 'normal' physiological range for various physical, blood, immune, (Chapter 6) and parasitological parameters (Chapter 2 and 5) as well as the physiological effects of common disease and environmental conditions on SBT (Chapter 2, 3, and 4). This knowledge can now be readily transferred to wild fisheries assessments.

Most commercial fisheries, including tuna, rely on their target species tendency to school, a behavior that also influences assessments of stock size and fisheries management. Therefore, changes in schooling

behavior are of great economic and ecological importance (Dell & Hobday 2008). Schooling, defined as a group of fish moving together with an element of organization (Fréon & Misund 1999), provides numerous advantages to its members including: (i) enhance predator vigilance and avoidance, (ii) increase feeding and breeding opportunities, and (iii) maximize energy efficiency (Shaw 1978). The Conservation Commission for Southern Bluefin Tuna (CCSBT) relies on stock assessments to manage the catch of southern bluefin tuna (SBT). Stock assessments use biological and fisheries data, such as stock age structure, fecundity, natural mortality, fishing pressure, sex ratio, growth rate, migratory habits, food preference, and estimates of total stock biomass. This information is used to generate a simulated computer model which informs fisheries managers on how to regulate a stock. But these assessments are only as accurate as the data supplied to them. In 1998, the CCSBT reviewed its list of fishery-based indicators, indicating a need to improve their accuracy (Gunn *et al.* 1998). In 2008, the CCSBT strongly recommended that the behavior of fish when schooling, an important aspect in fisheries management, should be incorporated into the model (Dell & Hobday 2008). However, while the schooling behavior of fish has been well described in relation to feeding, predation, and hydrological features, there has been little to no research completed on the effect of health status on schooling behavior (Mikheev 2009). Health status is known not only to affect schooling performance directly (Mikheev 2009), but also susceptibility to disease, growth (Barton 1997), and migration behavior (Guthrie & Kroger 1974). Differences in growth, fish migration patterns and disease susceptibility will have a considerable impact on accuracy of fish stock assessments. In 2008, the school size composition of SBT was analysed using archival data, and it was determined that between 1960 and 2000, fish schooling in Western Australia and the Great Australian Bight had increasing fork-length and the school was increasingly more variable in fish size over time (Dell & Hobday 2008). The authors hypothesized this may be due to reduced stock biomass, decreasing competition for food resources and reducing the number of fish within a year class or similar size group (Dell & Hobday 2008). But there are two problems with the data used for this hypothesis. First, only 52% of archival data were collected in a manner which fish could be assigned to a particular school. Therefore, during the 40 years over which fork length data were collected, information from merely 500 schools of SBT could be clearly identified. Second, between 1960 and 2000, data were increasingly collected on fish closer to shore. The closer to shore a fish population is the more potential

stressors it could be influenced by, for example more variable temperatures, pollution exposure, and pathogen exposure. While fish normally school by size, injury and disease may cause large variation in individual sizes within a school (Guthrie and Kroger 1974). In addition, injured and sick fish have been observed to seek shallow and warmer water to recuperate (Gunter and Ward 1961), thus fisheries managers were probably artificially increasing the incidence of sampling a sick fish when sampling in progressively shallower water. It is possible that the observed change in fish size within a school was related to something other than stock size, such as suboptimal health status of the small number of fish schools that were sampled. This can result in health and performance differences between cohorts prior to and during ranching (Chapter 2). If individual wild fish or schools of fish have drastically different health status, assessments based on data from limited numbers of schools could be drastically overestimating or underestimating stock size and behavior. Therefore, understanding the schooling behavior of sick versus healthy fish would allow fisheries researchers to design sampling regimes and computer models to describe more accurately and precisely the current status of fish populations. Presently, there has been no consideration for the health status of wild fisheries in stock assessments and management plans.

Additional research could also be completed to enhance the efficiency and productivity of ranching. The effect of various husbandry methods and physical data on performance could be teased out statistically utilizing data already collected by the ASBTIA over the past several decades of ranching: tow variables such as catch date, catch location, tow cage density, weather, and tow duration, stocking density, feeding regime and nutritional content, survival, site location, performance of nearby sites, physical variables such as current flow, site depth, suspended sediment load and weather during ranching. Exploiting the same data set, the ideal ranching location for could be described and used to evaluate new potential lease sites. Although data need to complete this analysis is currently collected by ASBTIA, this analysis fell outside the scope of this thesis.

Finally future research should aim for a greater understanding of *C. forsteri* infection and development of diagnostics and treatment. Chapter 5 describes several methods that could be utilized for greater accuracy and early detection of infection. Additionally these methods do not require lethal sampling.

Detection of antigen or fluke DNA using polymerase chain reaction (PCR) may be practical option. DNA of *Schistosoma mansoni* and *S. haematobium* can be detected in human stool, serum and urine using a highly repetitive, 121-base pair sequence (Hamburger *et al.* 2001, Pontes *et al.* 2002, Sandoval *et al.*2006). PCR and loop-mediated isothermal amplification (LAMP) using a specific primer for the 28S ribosomal DNA of *Schistosoma japonicum* was able to detect DNA from a single miracidium in the intermediate host snail within 1 day of infection (Kumagai *et al.* 2010). Non-lethal methods of diagnostics would allow for more frequent monitoring of infection and would aid in the development of treatment protocols.

Chapter 6 describes the first commercial use of Praziquantel in the treatment of SBT for *C. forsteri.* But there is much need for optimization of Praziquantel delivery, i.e. dose, timing of delivery, and frequency of treatment as well as long-term monitoring of Praziquantel use within several cohorts of SBT, numerous companies, and over several years. Alternative therapeutics should also be identified as security for resisstance development, occurance side effects, and to ensure optimal treatment. Hardy-Smith *et al.* (20120) found Closal® (Coopers Animal Health), Panacur 100® (Inveret Schering Plough Animal Health) and Fasinex 240® (Novartis Animal Health) to be ineffective. Other options may include Triclabendazol, Bithionol, or Nitazoxanide used for treating sheep liver fluke *Fasciola hepatica*, Albendazole used to treat Chinese liver fluke *Clonorchis sinensis* (Abramowicz 2004), Tetrachloroethylene used to treat large intestinal fluke *Fasciolopsis buski* (Rabbani *et al.* 1984) and small intestinal fluke *Metagonimus yokogawai* (Goldsmith 1978), Artemether (Ross *et al.* 2002) and Oxamniquine (Stelma *et al.* 1997) used as alternative treatments for *Schistosoma mansoni*.

Completing commercial scale research on bluefin tuna has proven to be a difficult task. Repeatedly research occurred under adverse conditions including extreme restrictions to research schedules, weather exposure, commercial confidentiality, and political agendas. Capture and towing of wild SBT left a significant degree of ambiguity in the commencement date of ranching for each cohort of SBT. In addition, confirmation on which private company or cohort of SBT the research was to be completed did not occur in advance and, at the worst, even occurred on the day of the first sampling. It was also common for commercial agendas to change even after commencement of a research project, causing the

researcher to abandon samples already collected and start again with a new cohort of SBT. It was very uncommon for commercial companies to allow a researcher to significantly vary their husbandry methods without significant justification (Chapter 2, 4, and 6). Furthermore, samples were often limited to commercial harvests (Chapter 3, 4, and 6), therefore the researcher must design their project around the typical commercial harvest schedule and varying sample sizes. Fortunately, a researcher with excellent relationships with the farm managers and superior negotiation skills can often influence which cohort of SBT are harvested on a certain day. Consequently, to overcome the obstacles associated with working on commercially ranched SBT, a researcher must develop close direct partnerships with the private companies, their managers, skippers, and deckhands, be flexible in their approach, have a firm grasp of statistics and be able to communicate their research at an industry level.

When conducting research on ranched SBT, experimental design needs to incorporate three unique characteristics of the SBT ranching industry: (1) one tow of wild fish is transferred into few, usually 2 to 4, grow-out pontoons (2) large variations between different tows of fish and how they respond to ranching (as outlined in Chapter 2), and (3) large individual variation between individual fish due to their wild origin. Upon arriving in the Tuna Farming Zone, one commercial tow of SBT is transferred into 3 commercial grow-out pontoons. While it may be possible to use a larger number of smaller pontoons, they would not be at commercial scale and therefore the experimental results could not be directly transferred into practice without additional experimentation. In addition, smaller pontoons are not supported by the commercial industry, as they require large additional expense in maintenance. However, due to the huge variation between individual SBT, the lack of replicate pontoons is not a problem. Unlike other aquacultured fish, wild SBT do not have identical rearing conditions prior to the initiation of ranching (described on pages 57-58), which influences how they respond to ranching. These individual differences are much larger than any artificial potential effects of containing SBT within different pontoons. Therefore large SBT level-replication must be considered before pontoon-level replication for experiments conducted on ranched SBT.

Another challenge was the degree of aggregation in *C. forsteri* infection of SBT (Appendix). Parasite aggregation occurs when most of the hosts in a population have few or no parasites, while only a few

heavily infected (Poulin 1993). The biological causes of parasite aggregation are: (1) spatial and temporal differences in the host or parasite population either due to seasonality or natural aggregations of parasite stages within the environment; (2) variation within the host population behaviorally, physiologically or immunologically which affects their susceptibility to infection (Shaw et al 1998). It is important to understand the degree of aggregation in order to understand infection dynamics and to evaluate effective treatment strategies (Poulin 1993). Aggregation affects the stability of a parasite population through mating dynamics, regulation of the abundance of host and parasite populations, and affects intensity of competition between parasites. For example, in a strongly aggregated parasite infection, one may choose to treat specific predisposed individuals compared to large scale mass treatment (Woolhouse, 1997).

A moderate to high degree of aggregation was found in *C. forsteri* infection in SBT (Appendix). Within the host, aggregation may influence transmission dynamics, the intensity and prevalence of infection, the distribution of disease symptoms, and effectiveness of different forms of treatment (Galvani 2003). Understanding the infection is aggregated explains the difficulties faced in researching the effects of infection (Chapter 5) and treatment (Chapter 6) and may be a reason for inconclusive results related to *C. forsteri* infection within this thesis as well as within the published literature (Aiken *et al*. 2006, 2008, Hayward *et al.* 2010, Hardy-Smilth *et al.* 2012, Norte dos Santos *et al.* 2012)

As all the research described performed in this thesis occurred at the commercial scale, it was able to result in immediate changes to the management of the commercial industry. Technology that was initially developed to study the effect of vitamin supplementation on SBT, baitfish injection (Chapter 2), was immediately adopted by ASBTIA as a method of delivering therapeutics. Insights into the acute mortality event (Chapter 3) highlighted a time where SBT need to be treated with care, were also immediately adopted by industry. Finally, the management strategies for *Cardicola forsteri* infection (Chapter 6) are both being commercially used. This thesis also resulted in large marketing opportunities for ASBTIA including (1.) evidence pontoon containment does not cause chronic physiological or immune stress on SBT (chapter 3) and (2.) ASBTIA was the first commercial industry in the world to scientifically evaluate the effects of offshore aquaculture.

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APPENDIX

C. FORSTERI AGGREGATION STATISTICS

There are three ways to measure aggregation (Poulin 1993):

1. Ratio of variance to the mean number of parasites in the host (s^2/mean). In this measurement, as aggregation level increases, so does the ratio. The limitation of this measurement is it varies by the mean number of parasites in a host as well as the host sample size. The ratio will equal zero when no aggregation is present and will equal the mean intensity of parasites at maximum aggregation (Wilson *et al.* 2002)
2. Parameter k of the negative bionomial distribution. The limitations of this measurement are: (a) it is not sensitive to the tail of the distribution, i.e. heavily infected hosts; (b) this is completely useless for comparisons between samples; (c) it varies by the mean number of parasites in a host as well as the host sample size. This measurement tends toward zero as aggregation increases and is large (~>20) when no aggregation is present (Wilson *et al.* 2002).
3. Index of discrepancy (D). Calculated by comparing the sum of the total number of parasites in a host to the total number of hosts in a sample. The limitations of this test are: (a) it is unreliable for small sample sizes; (b) it can only be used for comparison when samples are roughly the same size; (c) it cannot distinguish between randomly distributed and clumped populations. The index has a value of zero when there is no aggregation and a value of 1 when there is maximum aggregation.

For this analysis all three measurements were calculated using 'Quantitative Parasitology 3.0' (Reiczigel & Rózsa 2005). Data for this analysis came from Ch. 3, Ch. 4, and Ch.6.

Table 7 Summary of data and aggregation indices.

Year	Project	Ranching duration	N	%Prevalence (95% CI)	Mean intensity (95%CI)	S^2/mean	K	D
2010	Ch. 3 & Ch. 4 (Near shore)	4-9weeks	66	74.2 (62.19-83.49)	3.27 (2.69-3.96)	2.488	1.504	0.515
2011	Ch. 6	21 & 23 weeks	40	85.0 (70.22-93.25)	5.79 (4.56-7.44)	4.138	1.384	0.462

There was a moderate to high level of aggregation in *Cardicola forsteri* infection of SBT (Table 7). All three indicies were in agreement, therefore there is strong evidence these results are true.

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